A study of soluble and membrane-bound acetylcholinesterase present in mammalian brain and muscle.

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A STUDY OF SOLUBLE AND MEMBRANE-BOUND ACETYLCHOLINESTERASE PRESENT IN MAMMALIAN BRAIN AND MUSCLE

Thesis submitted

by

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ABSTRACT

Several methods for the solubilization of porcine brain acetylcholinesterase were investigated and the solubilized enzyme was subjected to electrophoresis and sucrose density gradient centrifugation.

Extraction with ethylenediaminetetraacetic acid (EDTA), solubilization with Triton X-100, Triton X-100/KCl and extraction with sodium cholate were inefficient in solubilization of the enzyme. Moderate enzyme yields were obtained by lysolecithin extraction but extraction with sodium deoxycholate provided the best enzyme yield.

The multiple molecular forms of acetylcholinesterase were resolved by starch block electrophoresis and electrophoresis on polyacrylamide rods and gradient slabs; and between two and five forms were obtained depending upon the method of solubilization. Molecular weight estimations showed that species having molecular weights of 120,000, 266,000 and 422,000 were present in both naturally soluble and membrane-bound acetylcholinesterase and these results were compared with those obtained by sucrose density gradient centrifugation.

The crude enzyme was purified by affinity chromatography (MAC-agarose column) and the purified enzyme was examined by electrophoresis on starch block and polyacrylamide gel.

Membrane-bound acetylcholinesterase in rat muscle was successfully solubilized with Triton X-100 and the molecular forms of the solubilized enzyme were resolved by electrophoresis on starch block and polyacrylamide gel. Nine molecular species were detected, with molecular weights ranging from 97,000 to 733,000. These were compared with the molecular weights obtained by sucrose density gradient centrifugation.

A study of the inhibition of muscle acetylcholinesterase by selected organophosphorus compounds was also made. The results show that the naturally soluble and detergent solubilized enzymes were sensitive to different inhibitors, and the detergent solubilized enzyme was more susceptible to inhibition than the naturally soluble enzyme.

The results are discussed in the light of the relationship of the enzyme with the membrane and the significance of its role with respect to the theories of nerve impulse transmission.

CONTENTS

SECTION I

INTRODUCTION

1. GENERAL PROPERTIES OF ACETYLCHOLINESTERASE

A. Historical Background

The current knowledge of the mode of action of acetylcholinesterase and its role in synaptic transmission has resulted from considerable research which is closely linked with work on acetylcholine and other well known neurotransmitters.

In 1867, Adolf von Baeyer was the first to synthesize acetylcholine and the synthesis was simply a link in his structure determination by derivatization and combustion analysis. (von Baeyer, 1867) However, it was Henry Dale who in 1913 first demonstrated that a particular ergot extract produced a very rapid but transitory fall in blood pressure when injected intravenously into cats. This observation was summarized in Dale's classic paper of 1914 (Dale, 1914), in which he introduced for the first time muscarine-like and nicotine-like actions of acetylcholine. Arthur Ewins who worked with Dale isolated the base responsible for the muscarine-like action from the ergot extract and showed that it was acetylcholine. (Dale & Ewins, 1914; Ewins, 1914). These observations thus triggered the beginning of research into acetylcholine and proteins related to the acetylcholine cycle.

Later, in 1915, Hunt showed that small doses of physostigmine potentiated the effects of acetylcholine in the heart and blood vessels (Hunt, 1915) and in 1918, Führer who worked with leech muscle showed that physostigmine potentiated the stimulating effect of acetylcholine by one million times. (Führer, 1917; 1918).

Dale (1914) had suggested the possibility of acetylcholine arising in the body and being very rapidly hydrolysed by the

tissues so as to avoid detection by known methods. Then in 1925, Abderhalden and Paffrath demonstrated that an enzyme capable of splitting a neurohumoral substance was present in the small intestine of horse and pig and so supported and confirmed Dale's suggestion. (Abderhalden & Pæffrath, 1925). Also, in 1926, Loewi and Navratil while working with isolated heart preparations demonstrated the cholinesterase-inhibiting properties of physostigmine and so provided further evidence of an enzyme responsible for hydrolysing acetylcholine. (Loewi & Navratil, 1926).

B. Classification

The discovery of Galehr and Plattner (1927) that serum and whole blood have different choline ester splitting activities led to the classification of cholinesterases.

Stedman et al (1932) prepared an enzyme from horse serum which was considered to be a specific esterase for choline esters and found that the esterase present hydrolysed butyrylcholine faster than acetylcholine. Glick (1938; 1939; 1941) investigated the substrate specificities of the serum cholinesterase and showed that the enzyme activity increased with increasing chain length of the acid moiety of the acylcholinesters, as it increased from C_2 to C_4, and the activity decreased thereafter. Vahlquist (1935) also showed that the cholinesters were not hydrolysed exclusively by human plasma, although these esters were hydrolysed more rapidly than others.

Alles and Hawes (1940) demonstrated the difference between human serum cholinesterase and human erythrocyte cholinesterase when they showed that the erythrocyte enzyme was inhibited by high concentrations of acetylcholine and that it hydrolysed

acetyl-β-methylcholine whereas the serum enzyme did not exhibit these properties. Mendel et al (Mendel et al, 1943; Mendel and Rudney, 1943a & 1943b) who worked on serum cholinesterases, investigated their sensitivities to inhibitors and substrates, and they classified the erythrocyte cholinesterase as true cholinesterase and serum cholinesterase as pseudocholinesterase. However, Nachmansohn and Rothenberg (1944; 1945) had a different system of classification and they named the erythrocyte enzyme as being specific cholinesterase (ChE) and the nerve enzyme as being acetylcholinesterase (AChE). Although the existence of two types of cholinesterases has been established, there is no clear cut classification of these enzymes as intermediate types of the enzyme have also been found. Rabbit, rat, chicken and frog plasma cholinesterases hydrolyse propionylcholine most rapidly while human, horse and dog plasma cholinesterases hydrolyse butyrylcholine at the greatest rate. The plasma cholinesterase of teleostian fish and elasmobranchs could be classified as an acetylcholinesterase on the basis of substrate specificity but this differs from acetylcholinesterase in its kinetic behaviour (Augustinsson, 1968). Another deviation from the general classification could be found in the enzyme of plaice muscle (Pleuronectes platessa) which demonstrated high activity with butyrylcholine but was inhibited by excess substrate, a characteristic of an acetylcholinesterase (Lundin, 1967a & 1967b).

Augustinsson (1963) defined cholinesterases as follows: "Cholinesterases constitute a group of esterases which hydrolyse choline esters at a higher rate than other esters, when hydrolysis rates are compared at optimum conditions regarding substrate concentration, pH, ionic strength, etc., using preparations free

from other esterases. All esterases which show this specificity are inhibited by 10^{-5}M eserine." This definition for cholinesterases seems to be satisfactory. However, the Enzyme Commission of the International Union of Biochemistry (1964) recommended that the cholinesterases be classified into two groups: those which hydrolyse butyrylcholine or propionylcholine (or their thio analogs) most rapidly and those which hydrolyse acetylcholine (or its thio analog) most rapidly. The former esterase was designated cholinesterase (acylcholine acyl hydrolase EC 3.1.1.8) and the latter as acetylcholinesterase (acetylcholine hydrolase EC 3.1.1.7).

In this thesis acetylcholinesterase will be abbreviated to 'AChE' and cholinesterase to 'ChE'. Also, cholinesterases will be used as a general term to include both types of enzymes. (Mc Intosh, 1973). The general properties by which acetylcholinesterase may be distinguished from cholinesterase are shown in Table I.1.

Aldridge (1953a & 1953b) and Augustinsson (1958; 1959a; 1959b and 1959c) recognized that certain esterases are different from cholinesterases. Therefore Aldridge (1953b) classified the serum esterases into two classes based on their sensitivity to inhibition by organophosphorus compounds: A-esterases (arylesterases) for esterases that are not inhibited and B-esterases (aliesterases) for esterases that are inhibited. He also designated the cholinesterases as C-esterases.

Another classification was introduced by Bergmann and Rimon (1957) whereby A-esterases are not inhibited by organophosphorus compounds; B-esterases are inhibited by organophosphorus

TABLE I.1

PROPERTIES AND NOMENCLATURE OF CHOLINESTERASES

	Acetylcholinesterase (AChE)	Cholinesterase (ChE)
Systematic name	Acetylcholine hydrolase	Acylcholine acyl-hydrolase
E.C. number	3.1.1.7.	3.1.1.8.
Optimal substrate	Acetylcholine	Butyrylcholine (propionyl or benzoyl choline for some enzymes)
Effect of excess substrate	Inhibition	No inhibition
Utilisation of acetyl- -methylcholine	Substrate	Non-substrate
Utilisation of butyryl- or benzoylcholine	Non-substrates	Substrates
Inhibition by BW62c47	Strongly inhibited	Very weakly inhibited
Inhibition by ethopropazine	Weakly inhibited	Strongly inhibited
Tissues and sources with high activity	Electric organ Human erythrocytes Brain	Serum Pancreas Heart Liver

In this thesis the above nomenclature will be adhered to throughout with the abbreviations 'AChE' and 'ChE' referring to the individual enzymes and 'cholinesterases' used as a general term to cover both types of enzyme.

compounds, and include the cholinesterases; and C-esterases are those which do not react with, nor are inhibited by organophosphorus compounds. The Enzyme Commission (1964), however, rationalized these divisions and recommended the classification of other esterases so that B-esterase became carboxyl ester hydrolase (EC 3.1.1.1); A-esterase became aryl ester hydrolase (EC 3.1.1.2); and C-esterase became acetic ester acetyl hydrolase (EC 3.1.1.6).

C. Occurrence in Nature

(i) Distribution in species The distribution of acetylcholinesterase has been investigated in considerable detail in connection with the physiological significance of the acetylcholine-acetylcholinesterase system. A number of reviews have demonstrated the diversity of species which contain the enzyme (Prosser, 1946; Augustinsson, 1948; Karczmar, 1963; Usdin, 1970). AChE has been found in almost all multicellular animals, in all vertebrates and in most invertebrates studied.

Early studies on vertebrates have shown that AChE was present in the rat, guinea pig, chick, dog and man. Very high concentrations of the enzyme was also found in the electric organ of the electric eel (Electrophorus electricus) and in varieties of torpedo fish (Torpedo marmorata, T. oscellata, and T. occidentalis). (Grundfest, 1957; Chagas & de Carvalho, 1961). These electric organs were reported to have the highest concentration of AChE found in any tissue (Marnay, 1937).

Among the invertebrates, acetylcholinesterase was found in annelids, molluscs, echinoderms, nemerteans, arthropods and coelenterates, but not in sponges, ctenophores or paramecia

(Prosser, 1946). The tunicate, Ciona intestinalis, was also found to contain AChE by Fromson and Whittaker (1970). It has also been reported that squid ganglion had the richest AChE content of any invertebrate tissue (Boell & Nachmansohn, 1940).

The existence of the enzyme in protozoa was doubted by Prosser (1946) and Augustinsson (1946) when they reported their findings but Seaman and Houlihan (1951) demonstrated that AChE was present in Tetrahymena geleii S. An enzyme which hydrolysed acetylcholine was also discovered in the myxomycete, physarum polycephalum (Nakajima & Hatano, 1962).

Acetylcholinesterase has also been found in the plant kingdom. Jaffe (1970) discovered AChE in the mung bean and Fluck and Jaffe (1974) while investigating plants containing AChE, found that the tomato and potato plants also contained AChE. Choline esters were also detected in certain types of fungi (Oury & Bacq, 1938).

The presence of AChE in bacteria is uncertain. Bernoulli and Bloch (1944) reported that bacteria, in all probability, did not contain the enzyme but Schaller (1942) found that the type I pneumococcus hydrolysed acetylcholine and a low hydrolytic activity for some bacteria was observed by de Prat (1945) and by Vincent and de Prat (1945).

(ii) General distribution in tissues In 1930, Plattner and Hintner studied the localization of acetylcholinesterase in tissue by pharmacological methods but their technique was not satisfactory. Marnay and Nachmansohn (1937; 1938) also investigated the presence and concentration of the enzyme in tissues. They demonstrated that AChE was present in remarkably high concentrations in all types of conducting tissue, nerve and muscle, vertebrate and

invertebrate, central and peripheral tissues, motor and sensory fibres, sympathetic and parasympathetic fibres.

In man and most other mammals, the enzyme is localized mainly in erythrocytes and in the nervous and neuromuscular systems. In fishes and birds, the AChE is mainly found in the serum. Apart from mammals, hydrolases of the AChE type are also found in the blood of the edible snail and in the venom of some snakes such as the cobra. However, of particular significance is the AChE content of the electric organ of the electric eel (Electrophorus electricus) and of varieties of torpedo fish. The enzyme has also been detected in the cranial ganglia of cuttlefish (Loligo pealii and Sepia officinalis).

D. Acetylcholinesterase in the Nervous System

(i) Localization in the neuromuscular system A major difficulty which arose in the chemical mediation theory of nerve impulse transmission was how acetylcholine (ACh), released by the nerve impulse, could be rapidly removed within the brief refractory period of skeletal muscles and ganglia. The solution to this problem was demonstrated by a series of well planned experiments on acetylcholinesterase, first carried out by Nachmansohn and his co-workers. In the frog sartorius muscle, the acetylcholinesterase concentration was found to be 300 per cent higher in the part containing nerve endings than in the nerveless pelvic end, and this was also observed in mammalian and other animal muscles. This difference was attributed to the high concentration of AChE at the nerve endings by Marnay & Nachmansohn (1937; 1938). They demonstrated that the concentration of AChE was high enough to support the assumption that ACh was the transmitter of impulses

across the neuromuscular junction.

Karnovsky (1961) studied the sarcoplasmic surface of rat skeletal muscle fibres and he showed that AChE was present in high concentrations along the postjunctional membrane. Furthermore, Karnovsky suggested in his publication that the AChE was synthesized locally. Bloom and Barrnett (1966) demonstrated by histochemical methods the association of the enzyme with most of the innervated membrane using eel electroplax. Autoradiography with $[^{3}H]$ and $[^{32}P]$ diisopropylphosphoryl fluoride (DFP) has shown AChE to be concentrated at least five-fold at endplates of the neuromuscular junction above the background nonjunctional-membrane region (Rogers et al, 1969). The separation of endplate from non-endplate regions of rat diaphragm muscle has led to biochemical estimates that 40% of the enzyme activity is associated with endplates, despite the relatively small area occupied by endplates (Hall, 1973).

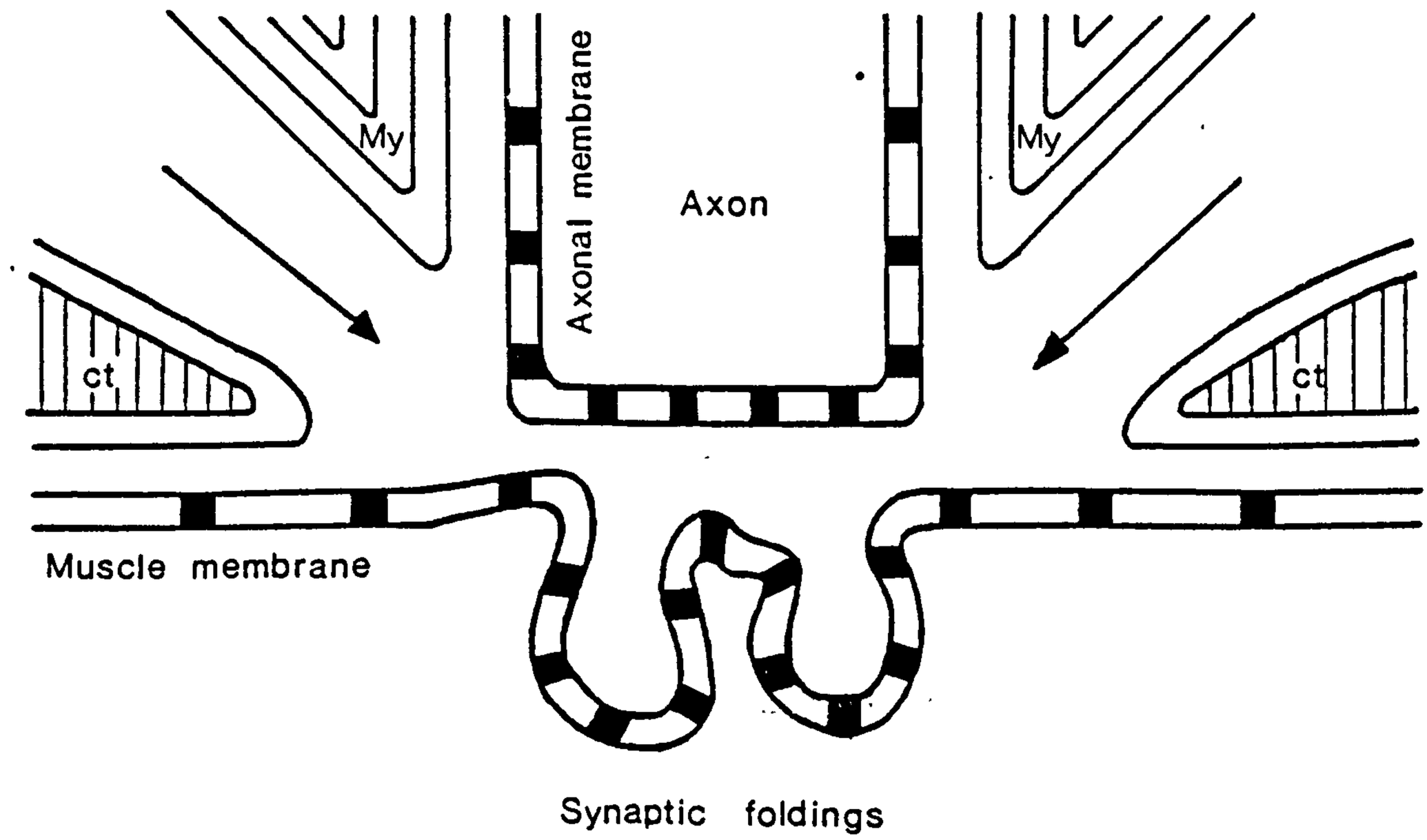
Friedenberg and Seligman (1972), in their review of the various histochemical procedures used for detection of acetylcholinesterase at the motor end plate pointed out that the sensitivity and specificity of the method used dictated the results obtained. They quoted the work of Adams et al (1969) who worked on nodes of Ranvier where it was uncertain whether the local staining in those regions was due to the actual presence of AChE or alternatively to the precipitation of thiocholine by copper present in the stain binding to nodal acidic mucosubstances.

Research on neuromuscular junctions by Betz and Sakmann (1971) and Hall and Kelly (1971) aroused the need to solve the true localization of acetylcholinesterase. These workers treated

neuromuscular junctions with collagenase and removed the ecto-lemmal sheath which they suggested acted like a 'cement' between nerve and muscle. They found that the AChE activity disappeared with the removal of the sheath and so they assumed that the enzyme was not a structural component. They were supported by further evidence that in non-innervated portions of muscle the enzyme was not released by treatment with collagenase. The cross section through a neuromuscular synaptic junction is shown in Fig. I.1.

(ii) Localization in the autonomic nervous system The precise localization of AChE in the autonomic nervous system is very difficult to determine since there is a low concentration of the enzyme relative to other esterases (Koelle, 1971). In some denervation experiments, it was found that following preganglionic denervation, the levels of AChE in the superior cervical ganglion of the cat fell dramatically (Koelle & Koelle, 1959). On the basis of its total disappearance following preganglionic denervation, the external or functional AChE was concluded to be confined almost exclusively to the presynaptic terminals. It was also proposed that the acetylcholine released by the preganglionic terminals acted initially at the presynaptic site as a positive feedback mechanism to amplify ACh release, and that the primary function of ganglionic AChE is the modulation of this process (Koelle, 1962). Similarly, as postulated by Burn and Rand (1959) for the adrenergic nervous system, Koelle also proposed that ACh facilitated the release of other transmitters at several non-cholinergic axonal terminals. While this working hypothesis could still be considered only tentative, it did provide explanation

Fig. I.1 Scheme of a Cross-Section through a Synaptic Junction between a Neuron and a Muscle Cell (Neumann and Nachmansohn, 1975)



The bars across the excitable membranes represent cross sections of the suggested basic excitation units (BEU); the density of BEU is assumed to be higher in the synaptic region than in the axonal parts. My, membrane layers of myelin protecting the axonal membrane; ct, protective layers (e.g. connective tissue) of the muscle membrane. The arrows indicate the sites of relatively easy access for external application of chemicals to the excitable membranes of nerve and muscle.

for the presence of moderate to high concentrations of AChE in many non-cholinergic neurons (Koelle, 1971).

In order to obtain direct evidence of the cytological localization of AChE in the superior cervical ganglion, an entirely new histochemical procedure that would approach in accuracy the degree of resolution afforded by electron microscopy was developed by Koelle et al (1974). Their results obtained by this procedure cast doubt on the limitation of the enzyme to the presynaptic membrane (Koelle et al, 1975). Examination by electron microscopy of sections of the cat superior cervical ganglion prepared by histochemical methods has revealed some striking differences in the localization of AChE from what had been concluded from the earlier light microscopic studies, as AChE was shown to be present at both the presynaptic and postsynaptic membranes. On the basis of the more direct evidence obtained by electron microscopy, Koelle et al (1975) attributed the disappearance of most of the ganglionic AChE that followed preganglionic denervation to not only the breakdown of the presynaptic terminals, but also to the loss of a trophic factor that was essential for maintaining the enzyme at the postsynaptic site.

(iii) Localization in the central nervous system Identification of the cholinergic systems present and the correlation of AChE levels with those of choline acetylase and acetylcholine was the basis of most of the research on the localization of AChE in the central nervous system.

Detailed investigations of cholinergic pathways has shown the presence of an extensive cholinergic innervation of the forebrain (Lewis & Shute, 1966; Lewis et al, 1967; Shute & Lewis,

1963, 1966). These studies demonstrated that many of the cholinergic fibres formed an ascending radiation from cells in the midbrain which were identical with the reticular activating system, and they terminated on cholinoreceptive cells in the striatum and cerebral cortex. The latter probably accounted for more than half of the cholinergic terminals in the forebrain (Silver, 1967).

Acetylcholinesterase has been shown to be associated with both classes of excitable membranes, conducting and postsynaptic, by histochemical criteria (Nachmansohn, 1971). In unmyelinated fibres, the localization of the enzyme in the axonal membrane was demonstrated by Schlaepfer and Torack (1966). Until recently, the myelinated nerve fibres was thought to lack AChE. However, Brzin (1966) treated the frog sciatic nerve axon membrane with Triton X-100 and he demonstrated the association of AChE with this membrane. Another interesting aspect of the enzyme localization, providing it is not a staining artifact, is the apparent localization of the enzyme in patches rather than being equally distributed along the membrane. This observation was reported in muscle cells of plaice (Lundin & Hellstroem, 1968), in lobster nerve membranes (De Lorenzo et al, 1969) and in squid giant axon (Brzin et al, 1965).

As early as 1955, AChE was shown to be present in the frog brain (Shen, Greenfield & Boell, 1955). Since then many studies on the investigation of AChE distribution in the brain and spinal cord of vertebrates have been carried out. Particularly high levels of AChE in the rat spinal cord and brainstem have been reported as a result of histochemical studies (Koelle, 1954; Giacobini, 1959; Navratnam & Lewis, 1970). Silver (1967) also

reported high levels of enzyme in rat cerebellum although its precise localization is uncertain.

Light and electron microscopic investigations on cultured nervous tissue have shown that neurons and glial cells grown in vitro retain similar morphological properties to those in vivo preparations (Bunge et al, 1965; Murray, 1965; Peterson et al, 1965; Hösli et al, 1973; Hösli et al, 1975). Furthermore, histochemical studies have shown that cultured neurons were able to store and/or to synthesize various enzymes which were known to be present in the nervous system in situ (Murray, 1971). The presence of AChE has been observed in cultures of the central nervous system of animals such as mouse, rat and chick (Kim & Murray, 1969; Hösli & Hösli, 1970, 1971; Minelli et al, 1971; Ieradi & Cataldi, 1972; Tischner & Thomas, 1973). Very few histochemical data are available on the presence of AChE in cultures of the human central nervous system (Geiger & Stone, 1962; Hösli et al, 1973, 1974).

The localization of AChE in cultures from different areas (spinal cord, brainstem, cerebellum) of rat central nervous system was also investigated by Hösli and Hösli (1970, 1971). They observed that although the number of AChE-containing neurons varied among individual cultures, there was a larger proportion of cells staining for AChE in spinal cord and brainstem cultures than in cerebellar cultures. The majority of neurons of spinal cord and brainstem also had a higher AChE content than cerebellar neurons.

In spinal cord cultures, groups of large intensely stained cells have been observed and they are thought to be motoneurons (Hösli et al, 1975). Many large neurons with a high AChE content

were observed in brainstem cultures and studies on sections of brainstem of newborn rats demonstrated that most of the AChE-staining neurons were found in the area of the hypoglossal nucleus and in the nucleus ambiguus. In cerebellar cultures, Hösli et al (1975) often observed large AChE-containing neurons with a pear-shaped cell body suggestive of Purkinje cells.

More recently, Knutsen et al (1975) have studied the sub-cellular distribution of AChE in five regions of pig brain (cerebral cortex, cerebellar cortex, caudate nucleus, thalamus and superior colliculi). They found that the rank order of the specific activity for AChE was: caudate nucleus, superior colliculi, thalamus, cerebellar cortex and cerebral cortex (having the lowest specific activity). The regional distribution of AChE in the frog central nervous system was investigated by Nistri et al (1975). They represented the central nervous system of the frog as six regions, namely, telencephalon, midbrain, rhomboencephalon, cervical spinal cord, thoracic spinal cord and lumbar spinal cord. They observed that in the frog brain the AChE activity was lowest in the telencephalon while activity in the midbrain and rhombo-encephalic regions were comparatively similar. In the spinal cord, Nistri et al (1975) reported that the AChE activity was fairly evenly distributed throughout the three areas studied.

E. Structural Composition

(i) Molecular weight and subunit structure The term 'multiple forms of the enzyme' was defined by the IUPAC-IUB (1971) as a broad term which applied to proteins possessing the same enzyme activity and occurring naturally in a single species. This form

of description is relevant to the enzyme AChE rather than the term 'isoenzyme' which essentially refers to multiple forms of the enzyme arising from genetically determined differences in primary structure instead of modification of the same primary sequence.

Molecular weight (MW) estimates vary considerably, probably owing not only to the different methods and conditions used, but also to the various degrees of aggregation which arise from different treatments. Most molecular weight and subunit determinations of AChE have been performed on the electric eel enzyme.

In the early 1940s, ultracentrifugation studies of purified AChE from Electrophorus electricus provided considerable data on the MW of AChE. Rothenberg and Nachmansohn (1947) and Lawler (1963) suggested the existence of AChE in several forms on the basis of sedimentation data. In the 1960's several estimates of molecular weight for AChE were reported. Lawler (1961) estimated the MW of AChE to be 240,000 while in a later publication (Lawler, 1963) she described studies on large aggregates and also estimated the MW of a smaller species to be 330,000. Kremzner and Wilson (1963) reported the MW value to be 230,000 and Leuzinger et al (1969) arrived at a value of 260,000. Several other laboratories have shown that AChE in solution existed in different multiple molecular forms (Hargreaves et al, 1963; Massoulié & Rieger, 1969; Dudai et al, 1973).

An important new development started with the observation of Massoulié and Rieger (1969) that there were three different species of AChE in fresh electric tissue extracts. Most homogeneous preparations obtained from toluene-treated tissue were characterized by a single band with a sedimentation coefficient

of about 11 S. However, when AChE was extracted from fresh electric tissue, using high ionic strength (1M NaCl), three molecular forms of enzyme were obtained in sucrose gradient centrifugation with sedimentation coefficients of 8 S, 14 S and 18 S. These enzyme species were converted to the 11 S form by treatment with trypsin or other purified proteolytic enzymes, or on storage of crude enzyme extracts from electric tissue slices stored in toluene (Massoulié et al, 1971; Rieger et al, 1972 a, b). Dudai et al (1972b) purified fresh electric tissue extracted with high ionic strength, 1M NaCl, by affinity chromatography using the ligand 1-methyl-9-aminoacridinium (attached to the resin matrix via the 9-amino group). They eluted the enzyme from the column with decamethonium bromide and the highly purified enzyme showed the same species differences as those described by Massoulié and his co-workers in crude extracts. Dudai et al (1973) determined the MW values for the 14 S and 18 S species by sedimentation equilibrium and obtained values of 780,000 and 1,000,000, respectively, for the two species. Similar MW values for the 14 S and 18 S species were estimated by Bon et al (1973) on the basis of measured values of the Stokes radius, the partial specific volume, and apparent sedimentation coefficients. They also estimated the MW for the 8 S species to be 430,000 and proposed that the 8 S, 14 S and 18 S forms were aggregates which included varying numbers of 11 S tetrameric species. Dudai and Silman (1973) estimated the MW for the 11 S species by sedimentation equilibrium and found the MW value to range from 320,000 to 350,000. Rosenberry et al (1974) estimated the MW of the 11 S species to be about 280,000 to 300,000, while Taylor et al (1974) found that the MW of an

11 S form extracted and purified from Torpedo electric tissue was 335,000, either by sedimentation equilibrium or by sedimentation and diffusion coefficients analysis.

Acetylcholinesterase can be isolated from various sources in a number of multiple molecular forms, differing in molecular weights, Stokes radii, sedimentation coefficients, and isoelectric properties, depending on the method of solubilization. According to early procedures (Rothenberg & Nachmansohn, 1947; Lawler, 1963; Kremzner & Wilson, 1963; Leuzinger & Baker, 1967), the electric eel (Electrophorus electricus) enzyme was solubilized by prolonged autolysis under toluene and consequent extraction of lipids. Using conventional chromatographic procedures and gel filtration, a globular tetrameric enzyme was obtained (8 to 12% of the activity present in the extract). Leuzinger et al (1968) crystallized this enzyme and later in 1969, Leuzinger et al demonstrated that the enzyme comprised two different subunits of equal molecular weight. Massoulié and Rieger (1969) designated the purified form of AChE as G_p and since the introduction of affinity chromatography (Berman & Young, 1971), the G_p form could be purified more efficiently, yielding up to 70% in the final product.

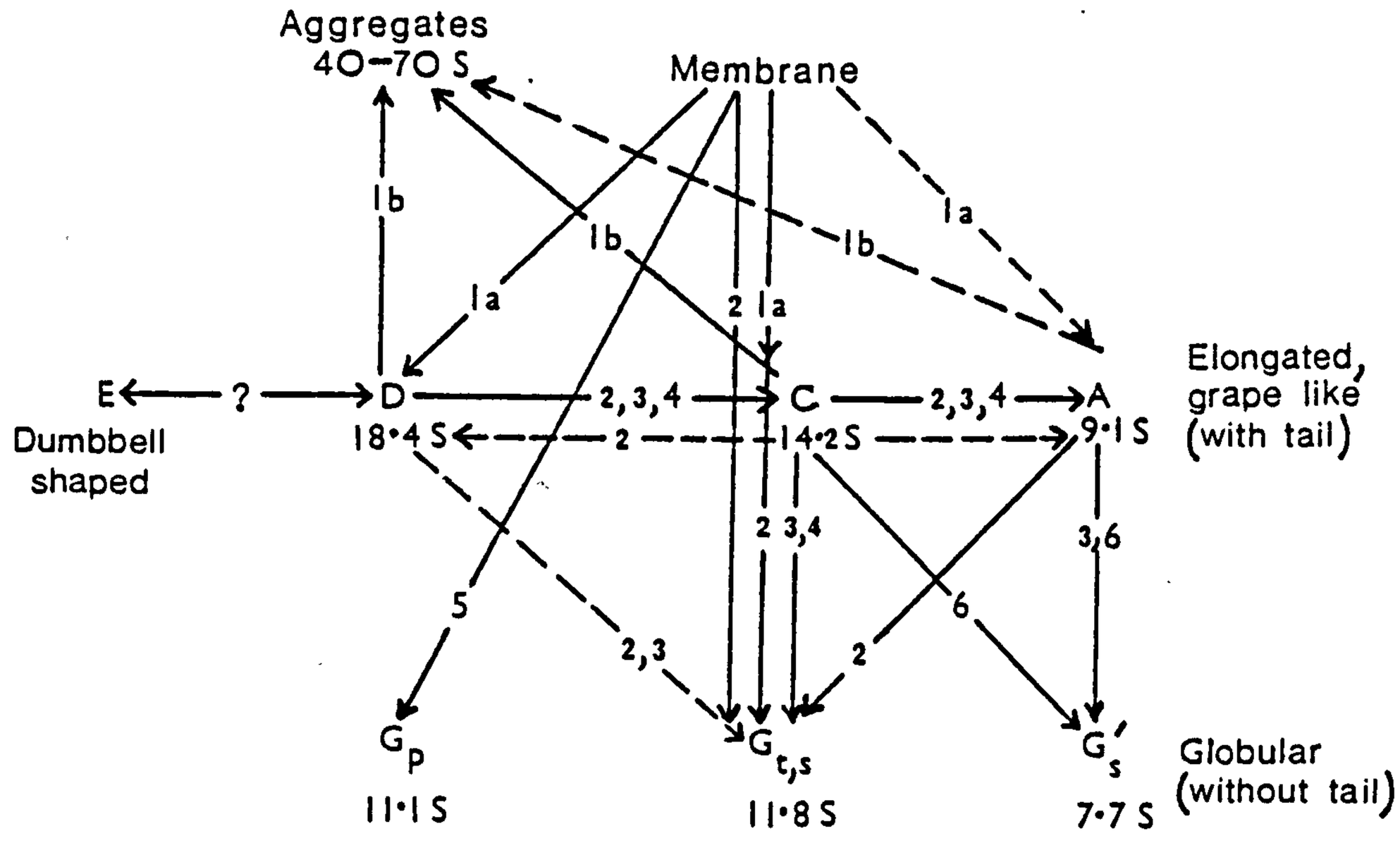
Grafius and Millar (1965, 1967) demonstrated that even after prolonged toluene treatment, other molecular forms of the enzyme could be obtained. These forms had sedimentation coefficients of 14 S and 18 S and aggregated to 65 S particles when dialyzed against buffers of ionic strength below 0.1. Massoulié extended this work and he showed that membrane-bound AChE, in the absence of proteolytic solubilization procedures, could be obtained in highly asymmetric forms termed A, C and D. Electron microscopic

studies of these forms showed that they appeared as grape-like structures, being composed of a tail with clusters of AChE subunits attached to it (Massoulié & Rieger, 1969; Massoulié et al, 1970a, 1971; Rieger et al, 1973b; Bon et al, 1973; Dudai et al, 1973). The non-aggregated forms of AChE do not have the tail and are thus more globular. Dudai said that it was tempting to speculate that the tail structure was involved with fixing the enzyme to the membrane although there is no evidence yet. A dumbbell-shaped species of AChE has also been observed and it seemed to be composed of two clusters of AChE subunits joined by a tail-like structure (Dudai et al, 1973; Wermuth & Brodbeck, 1972).

As mentioned earlier, the mode of solubilization of the enzyme or treatment of the solubilized forms could determine to a large extent the species of AChE obtained. Proteolytic treatment yielded the globular forms G_t and G_p, which did not have the typical tail-like extension present in forms A, C and D (Massoulié et al, 1970b; Dudai et al, 1972a). Solubilization by Triton X-100 and/or sonication yielded a dimeric globular form designated as form G'_s (Massoulié et al, 1971; Millar et al, 1973). Research on the nature of the various forms of AChE has also been confirmed and extended by other investigators (Dudai et al, 1972b; Dudai et al, 1973; Berman, 1973). The methods of solubilization of the enzyme from the membrane and the interconversions among the various forms were described diagramatically by Wermuth, Ott, Gentinetta and Brodbeck (1975) and this is shown in Fig. I.2.

Many enzymes were found to be formed by varying numbers of subunits. These subunits may have different functions; there are regulatory and catalytic subunits that exhibit allosteric activations

Fig. I.2 Ways of Solubilization of the Enzyme from the Membrane and the Interconversions Among the Various Forms (Wermuth, Ott, Gentinetta and Brodbeck, 1975)



1a, High ionic strength; 1b, low ionic strength; Ca^{2+}; 2, tryptic digestion; 3, sonication; 4, spontaneous; 5, autolysis, lipase treatment; 6, Triton X-100.

The dashed arrows indicate proposed pathways for the interconversion among the various forms, the solid lines give the ways of solubilization.

and inhibition, positive and negative cooperativity, feedback inhibition, and other characteristics (Moyed & Umbarger, 1962; Monod et al, 1963, 1965; Umbarger, 1964; Stadtman, 1966; Koshland, 1970).

It was only during these last few years when homogeneous proteins of AChE became readily available that the subunit structure of the enzyme was studied in several laboratories (Froede & Wilson, 1970; Dudai & Silman, 1971). Since many molecular forms with different sedimentation coefficients appear to be clusters with varying numbers of 11 S forms attached, Rosenberry et al (1974) investigated extensively the subunit composition of the 11 S species into which the other forms are readily transformed by proteolytic enzymes or by autolysis in toluene-treated material. This species may be the molecular form which is most common although it is possible that it exists in membranes in various aggregated forms.

Rosenberry et al (1972) and Chen et al (1974) purified the 11 S enzyme by affinity chromatography, from extracts of toluene-stored electric eel tissue. They also employed polyacrylamide gel electrophoresis in 1% sodium dodecyl sulphate (SDS) and gel exclusion chromatography in 6 M guanidine chloride. Their data indicated that the active enzyme was a tetramer composed of 4 identical subunits and each subunit was shown to contain one active site on the basis of analysis of ^{32}P phosphorylation by DFP and cyanogen bromide fragment compositions. These results were consistent with those of Rosenberry and Bernhard (1971) in which the active sites were shown to be kinetically homogeneous. The native enzyme appears to exist as subunit dimers with a covalent intersubunit linkage that involves disulphide bonding and the data

support a tetrameric structure with a dimer of dimers $(\alpha_2)_2$ (Rosenberry et al, 1974). Earlier, Leuzinger et al (1968) had centrifuged AChE in guanidine and subjected it to SDS gel electrophoresis. They concluded that the enzyme was a tetramer (MW 240,000) consisting of two different subunits, thus giving a dimeric hybrid $(\alpha\beta)_2$. Dudai and Silman (1972) also found two different subunits in the enzyme, one with a MW in the range 82,000 - 100,000 and the other with a MW of about 59,000, the tetramer having a MW of 320,000 - 350,000. These results were in agreement with those observed by Powell et al (1973) who referred to the subunits as light (l) and heavy (h) and denoted the tail structure as 'q'. On the basis of these observations, it is possible to obtain a range of molecular weights for the enzyme by using different permutations of the different subunits and Wermuth et al (1975) have recorded the different combinations of subunits (Table I.2 and Table I.3). On the basis of results from more recent studies by Rosenberry et al (1974), the earlier suggestion by Leuzinger et al (1969) that the enzyme had two different polypeptide chains giving a dimeric hybrid $(\alpha\beta)_2$ was unequivocally ruled out by Nachmansohn (1975). Rosenberry et al (1974) suggested that the dimeric hybrid model had been based on observations of the subunits at several stages of proteolytic cleavage.

Electron microscopic studies by Cartaud et al (1975) showed that the higher molecular weight forms of AChE were composed of aggregations of tetrameric units. They suggested that species D of the enzyme was composed of 3 tetramers which were joined by 3 filaments projecting from the tetramers and the filaments eventually came together to form a tail which had an α-helical structure.

Table I.2

Subunit Composition of Acetylcholinesterase

Reference	Molecular weight			Oligomer	
	Oligomer	Subunit	Condition[a]	Number of subunits	Number of active sites
Leuzinger, Goldberg and Cauvin (1969) & Leuzinger (1971)	260,000	64,000	Gu-HCl, Merc	4	2
Rosenberry, Chang and Chen (1972)	245,000	-		3	3
Froede and Wilson (1970)	224,000	49,000	Gu-HCl, Merc	4	4
Millar and Grafius (1970)	259,200	42,000	Gu-HCl, DTT	6	6
	Protomer			Protomer	
Froede and Wilson (1970)	102,000		Gu-HCl	2	2
Massoulie, Rieger and Bon (1971)	155,000	74,000 62,000	SDS	2	-
Dudai and Silman (1971)	160,000	90,000 64,000	SDS SDS, Merc	2	-
Millar, Grafius, Palmer and Millar (1973)	134,000	65,000	SDS	2	-

[a]Gu-HCl : guanidine-HCl; DTT : dithiothreitol; Merc : mercaptoethanol; SDS : sodium dodecylsulphate. (Wermuth, Ott, Gentinetta, and Brodbeck, 1975)

Table I.3

Properties of Elongated and Globular Forms of Acetylcholinesterase

Form	Subunit composition	Molecular weight (daltons)	Specific activity (IU/mg.protein)	Stoke's radius (nm)	Sedimentation coefficient (S)	Occurrence in EM
Elongated						
A	h_2l_2q	430,000		12.4	8.5-9.1	grape-like
C	h_4l_4q	780,000	14,300[a]	14.4	14.2	grape-like
D	h_6l_6q	1,110,000		15.0	18.4	grape-like
E	h_8l_8q	1,560,000[b]	13,300	17.0	-	dumbbell-shaped
Globular						
G_p	h_2l_2	260,000	15,200	8.2	11.1	tetrameric
G_t	h_2l_2	290,000	-	-	11.8	tetrameric
G'_s	hl	155,000	-	6.4	7.7	dimeric

[a] Average value for forms A, C, and D.

[b] Calculated for the subunit composition h_8l_8q

(Wermuth, Ott, Gentinetta, and Brodbeck, 1975)

Research on AChE isoenzymes from other tissues such as erythrocytes, diaphragm and brain have been carried out but the results have not been as enlightening as those from electric tissue AChE. Bovine brain AChE was isolated by Hollunger and Niklasson (1973) and the enzyme had a MW of 80,000. This enzyme was found to aggregate on storage but if it was prepared in DEAE-Sephadex-A25, the enzyme did not aggregate thus suggesting the removal of an aggregating factor. Similarly, Viana et al (1974) using the same tissue found that the smaller species of the enzyme (MW 120,000) aggregated through an intermediate form to a larger molecule (MW > 500,000). Studies on porcine brain AChE showed that the enzyme existed in several forms, the most frequently found forms being 60,000, 130,000, 198,000, 266,000 and 350,000 (McIntosh & Plummer, 1973). Plummer et al (1975) extended this work and they again found that the most common species present in the naturally soluble and membrane-bound enzyme had MW values of 68,000, 264,000 and 365,000. Ott et al (1975) demonstrated that the AChE subunit from Triton X-100 solubilized human erythrocyte existed in several forms differing in net charge but having similar molecular weights. They also showed that removal of the detergent led to the formation of various molecular forms of the enzyme. In contrast, Wright and Plummer (1973) found that solubilization of human erythrocyte AChE using Triton X-100/KCl mixtures resulted in various molecular forms of the enzyme.

Electron microscopic studies have demonstrated the existence of a protein tail which projected from an assembly of several subunits and so it is tempting to suggest a multiple molecular aggregate of enzyme being attached to the membrane via the tail.

The exact nature of the enzyme in the membrane, whether it exists as a monomer or a multiple molecular aggregate, is still uncertain. Recently, Levinson and Ellory (1974) have demonstrated the existence of the enzyme as a monomer (MW 75,000) in the membrane and explained that multiple forms observed were probably aggregated forms of the monomer. Powell et al (1973) have shown that the enzyme is a glycoprotein and this may be particularly significant since many surface membrane proteins have been proved to be glycoproteins. The glycoprotein nature of AChE would correlate well with its anticipated location at the surface of the synaptic membrane. Rieger et al (1973a) and Sihotang (1976) have also demonstrated that phospholipid is involved with the enzyme, probably in the aggregation phenomenon.

(ii) Equivalent weight and number of active sites The equivalent weight of a protein is given by its grams per mole of active sites. The moles per litre of enzyme active sites (enzyme normality) can be determined for any enzyme solution provided that a sufficiently sensitive titrating agent is used.

Several titrants which have been used with AChE have operated by acylating the active site serine and thereby provide high specifity for the catalytic site. Radioactive phosphorylating agents such as $\left[{}^{32}P\right]$ diisopropylphosphoryl fluoride have been employed to determine enzyme normalities (Michel & Krop, 1951) since the phosphorylated AChE is quite stable. Later, Lawler (1961) and Kremzner and Wilson (1964) used the stoichiometric loss of activity as a function of phosphorylation by N,N-Dimethyl-S-(diethylphosphoryl) thioethanolamine to determine the enzyme normality. However, carbamoylated AChE is less stable and radioactive titration is

impractical. Rosenberry and Bernhard (1971) and Mooser et al (1972) introduced the fluorogenic agent 1-methyl-7-dimethylcarbamoyloxyquinolinium iodide, which exhibited diminished quantum yields when associated with the enzyme, and obtained the enzyme normality by this procedure. In addition, many fluorescent ligands are observed to be totally quenched when they bind to the enzyme active site (Mooser et al, 1972) and also this titration should be applied with caution in crude-enzyme extracts since nonspecific binding of the ligands to other components may contribute to the observed quenching.

The equivalent weight of AChE was calculated to be 76,000 g/mole of active sites by Rosenberry (1975) and this weight is precisely the subunit molecular weight of the eel 11 S enzyme. The number of active sites per molecule is given by the molecular weight divided by the equivalent weight which is simply the number of subunits per molecule. Hence, for the eel 11 S enzyme, the number of active sites is 4 per molecule, as reported by several groups (Kremzner & Wilson, 1964; Froede & Wilson, 1970; Rosenberry & Bernhard, 1971; Mooser et al, 1972; Chen et al, 1974).

F. Biological Function of Acetylcholinesterase

The importance of the role of AChE in biological processes, such as controlling ionic currents in excitable membranes is demonstrated by effects of anticholinesterase agents on electrical activity. Anticholinesterases inhibit enzyme catalysis and are usually acylating agents which phosphorylate or carbamoylate the esteratic site serine. The action of anticholinesterases is most obvious at synapses in neuromuscular junctions and autonomic

ganglia (Goodman & Gilman, 1955), because the excitable membranes at these sites appear to be associated with relatively few protective structural barriers and hence are rendered more accessible to the drugs. Anticholinesterase effects may also be demonstrated on axons provided the structural conditions are favourable, for example on the Ranvier nodes of single axons of the frog sciatic nerve and on certain lobster axons. The clinical and pharmacological effects of anticholinesterases applied to the whole organism generally result from their effect on synapses. Pronounced anticholinesterase exposure can lead to death from respiratory and circulatory failure. Owing to the importance of AChE considerable effort has been spent in developing anticholinesterases with the appropriate species selectivity for use as insecticides. There is no well-defined role ascribed to the AChE in erythrocyte membranes although studies have been directed toward possible roles in cation transport and membrane rigidity (Taylor et al, 1952; Giberman et al, 1973; Heustis & McConnell, 1974; Nachmansohn, 1975).

(i) Relationship of acetylcholinesterase and acetylcholine receptor

In the early stage of development of the hypothesis postulating ACh to be a neurohumoral transmitter, the target of this transmitter was not yet discussed. It was suggested that the transmitter stimulates the second cell (nerve or muscle) in a way which was not specified; the inactivation of ACh was specifically due to the hydrolysis by an esterase (Dale, 1937). The notion of a receptor of ACh emerged only in the last twenty years, particularly when the effects of a great variety of inhibitors of AChE was studied due to the widespread interest in organophosphates. It became apparent to several investigators that it was difficult to explain some of the

pharmacological actions and electrophysiological observations simply in terms of enzyme inhibition (Wescoe & Riker, 1951; Riker, 1953). In the 1960's, an increasing number of observations with inhibitors of AChE were reported by many investigators, supporting the assumption of an action of some of these inhibitors on a receptor as distinct from and preceding that on the enzyme.

The notion of a specific ACh-receptor protein was first postulated by Nachmansohn (1952). There was no experimental proof for this assumption, it was based on the notion that only a protein has the ability to recognize with a high specificity a ligand such as acetylcholine.

Owing to the fact that it is possible to isolate and study in vitro 'the cholinergic receptor', the research on such receptors has been widely extended. All cholinergic receptors respond to acetylcholine although they may require different conformations from this substance (Pfeiffer, 1956; Pauling & Petcher, 1970; Baker et al, 1971). Nicotinic ACh receptor is found in postganglionic parasympathetic receptors, in cerebellar cortex and is characterized by tetramethylammonium ions blocking ACh effects. The muscarinic receptor is found in postganglionic parasympathetic receptors, in cerebral cortex, and in caudate nucleus; the ACh effect is blocked by atropine. The intermedian receptor is found in brainstem and thalamus. Drug studies have shown that the nicotinic receptor greatly lacks stereospecificity (Barlow, 1965), while the muscarinic receptor is highly stereoselective (Armstrong et al, 1968).

When discussing recent advances in the isolation of cholinergic recognition sites (ChR), a name more adequate for the isolated

material than acetylcholine receptor (AChR), it is essential to remember that the isolated material may differ in certain properties depending upon (a) the type of tissue used for purification and (b) the method of purification, particularly if biospecific chromatography is used. It must also be remembered that in addition to AChR there are other well-known molecules with sites that recognize ACh such as the cholinesterases (ChE). In the nervous system, AChE is located where AChR is, for example, in the postsynaptic membranes of nerves, in electric organs, and in muscle endplates. AChR differ from ChE in that they cannot hydrolyse ACh, but there is as yet no proof that the binding sites and the peripheral (allosteric) sites of ChE really differ from AChR. Heilbronn et al (1972) and Heilbronn & Mattsson (1974) have shown that solubilized Torpedo ChR is heterogeneous or slowly changes its properties with time; two components differing in affinity for Naja n. siamensis neurotoxin have been seen. Also, Eldefrawi and Eldefrawi (1973) have also observed that some of the ChR isolated from Torpedo electric tissue bound D-tubocurarine and Elapide snake neurotoxins but little ACh or decamethonium. It is not yet known if these components are different multiples of the same subunits, or the same molecule with partly changed properties. Bourgois et al (1972) have also demonstrated that extrasynaptic as well as synaptic receptors are found in electric tissue and Waser (1970) suggested that they may have different properties.

Early efforts to characterize the cholinergic receptor chemically were mostly rather unsuccessful. However, studies by Karlin (1969) showed that functional disulphide bonds are necessary for receptor activity and recent amino acid analysis of isolated

receptor proteins has confirmed the presence of small amounts of half cystine in the molecules.

The combined action of the enzyme and the receptor in controlling membrane ion currents is demonstrated by the close association of these two glycoproteins in excitable membranes (Bloom & Barrnett, 1966; Brzin, 1966; Rogers et al, 1969; Nachmansohn, 1971; Miledi & Potter, 1971; Hall, 1973). The membrane association of the two glycoproteins in electric organ differs in that AChE belongs to the category of extrinsic or peripheral membrane proteins while the receptor has characteristics of intrinsic or integral membrane proteins. While this distinction is not clear in mammalian nerve and muscle by the apparent detergent requirement for extraction of most particulate AChE species, the continued solubility of these extracted mammalian species in aqueous solution free of detergent justifies the general classification of the enzyme as an extrinsic membrane protein in these tissues.

Although the isolated enzyme and receptor are distinguishable species, several investigators have obtained evidence that levels of enzyme and receptor in the membrane are regulated by a common genetic-control mechanism during growth and differentiation in tissue culture (Blume et al, 1970; Simantow & Sachs, 1973; Prives & Patterson, 1974). There is also evidence that the enzyme and the receptor appear to be present at about equal concentrations in synaptic regions (Changeux et al, 1970; Karlin et al, 1971; Miledi & Potter, 1971; Porter et al, 1973) and this has led one to consider whether they might be linked with a fixed stoichiometry in a protein complex similar to those involving the respiratory assembly of the inner mitochondrial membrane (Lardy & Ferguson, 1969), the fatty-

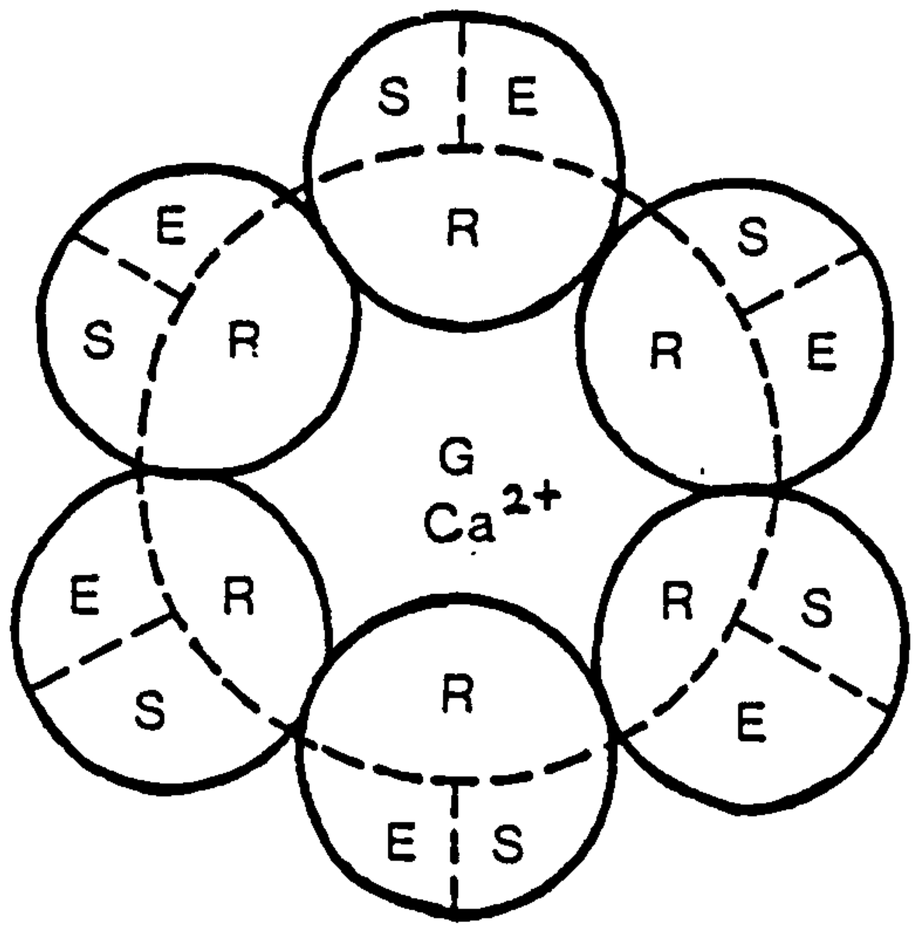
acid synthetase particle (Ginsburg & Stadtman, 1970), and the myofilament system in muscle (Huxley, 1969). Neumann et al (1973) have proposed a general model of nerve excitability, postulating the involvement of these two glycoproteins in such a protein complex termed a 'basic excitation unit' (BEU). (Fig. I.3).

Analyses of membrane-fragment distributions formed by sucrose density gradient centrifugation have provided data concerning possible complex formation between molecules of enzyme and receptor. These membrane-fractionation experiments have to be interpreted carefully because extraction of particulate enzyme during homogenization, followed by aggregation or nonspecific adsorption to other membranes, could easily distort the physiological enzyme distribution (Dudai & Silman, 1973).

The spatial relationship of the enzyme and receptor has also been studied by autoradiography of mouse sternomastoid-muscle endplates (Salpeter et al, 1972; Fertuck & Salpeter, 1974). From the $[^{3}H]$ DIP-acetylcholinesterase labelling patterns it was concluded that the enzyme is relatively uniformly distributed along the junctional folds of the postsynaptic membrane. Betz and Sakmann (1971) suggested that the enzyme may be anchored in the ectolemmal sheath which surrounds the muscle fibre. Recent studies by Fertuck and Salpeter (1974) suggests a somewhat different distribution for endplate receptor labelled with $[^{125}I]\alpha$-bungarotoxin; the receptor appeared localized on the 'top' surface of the postjunctional folds, that is, in membrane areas nearest the axonal membrane. In addition, the estimated membrane density of the receptor molecules in these regions approach predicted values for a receptor monolayer. High resolution electron micrographs for

Fig. I.3 Scheme of the Acetylcholine-controlled Gateway, G (Neumann and Nachmansohn, 1975)

(a)



(b)

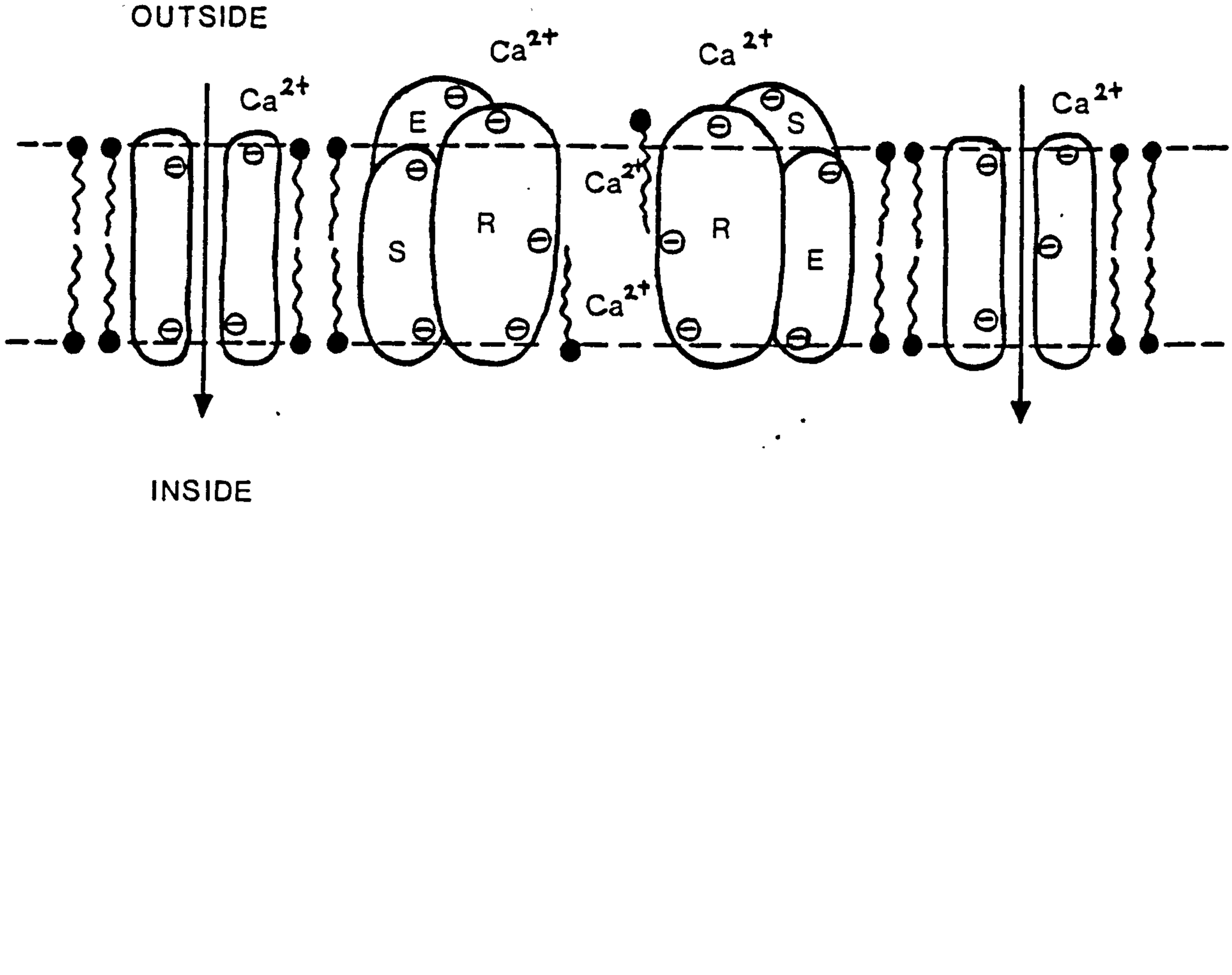


Fig. I.3 Scheme of the Acetylcholine-controlled Gateway, G (Neumann and Nachmansohn, 1975)

(a) Basic excitation unit (BEU) containing in this example 6 SRE-assemblies, viewed perpendicular to the membrane surface. S, acetylcholine-storage site; R, acetylcholine-receptor protein, E, acetylcholinesterase.

(b) Cross section through a BEU flanked by two units which model ion passages for K^+ ions; the arrows represent the local electrical field vectors due to partial permselectivity to K^+ ions in the resting stationary state. The minus signs, $\ominus$, symbolize negatively charged groups of membrane components.

receptor-rich membrane fragments from torpedo support locally high receptor densities, in which the receptor may comprise 10-20% of the membrane protein present and they exhibit a lattice organization of doughnut-shaped particles (Cartaud et al, 1973; Potter, 1973) which resemble particles obtained in highly purified, detergent-solubilized receptor preparations. These biochemical, autoradiographic and electron microscopic studies appear to call into question a protein assembly involving a molecular interaction between the enzyme and the receptor; the receptor, however, appears to form membrane patches, perhaps by self-aggregation, which exclude most other membrane proteins and it has been suggested that such stabilized patches could prevent translational membrane diffusion of the receptors away from the synapse (Rosenberry, 1975).

(ii) The enzyme at synapses

(a) Identification as a distinct molecular species The enzyme is quantitatively concentrated on or near postsynaptic plasma membrane. Studies on rat diaphragm suggest that AChE which was associated with endplate membrane can be extracted as a species distinct from the enzyme in extrasynaptic membrane (Hall, 1973). The anatomy of the rat diaphragm allows sarcolemma containing endplate (probably presynaptic nerve terminals as well) to be separated from nonendplate sarcolemma by dissection and analysis of the enzyme species isolated from these two regions have shown that the 16 S species is localized almost exclusively in the endplate region, by inference, in the endplates themselves. In contrast, the 4 S and 10 S species are extracted from both regions and only the 4 S and 10 S species are extracted from phrenic nerve trunks.

Following denervation, the 16 S species, and to a lesser extent the 10 S species, disappear from the endplate region. Hall (1973) has also demonstrated by electrophysiological and histochemical studies that the enzyme is present in denervated muscle endplates, although the amount of endplate enzyme is decreased.

Albuquerque et al (1968), Betz and Sakmann (1971), and Hall and Kelly (1971) have shown that muscle endplate enzyme may be selectively detached and removed by protease treatment. Treatment with collagenase (or a protease contaminant) yielded a 10 S species of the enzyme and collagenase treatment of the 16 S species in solution after extraction at high ionic strength led to its conversion to a 10 S species. Transformations of this type are also evident in the electric organ tissue and if the knowledge about the molecular structures of the corresponding electric organ enzyme species is applied to the data from rat diaphragm, it may lead to the suggestion that the aggregative 14 S and/or 18 S electric organ species reflect synaptic-membrane species.

(b) Aggregation as a model for membrane association The suggestion that the molecular affinities promoting aggregation of the 14 S and 18 S eel electric organ species at low ionic strength being related to the affinities involved in the association of the particulate enzyme with the membrane in vivo has been put forward by Dudai and Silman (1973, 1974b). Grafius et al (1971) and Dudai and Silman (1974b) have also proposed that the aggregate itself is the basis of a physiological enzyme 'matrix', which could prevent translational membrane diffusion of the enzyme from the synaptic region.

It has been demonstrated by some investigators that high ionic-strength buffers or maleylation solubilized the 14 S and 18 S species of the eel enzyme (Dudai & Silman, 1974b). When solubilized by maleylation, these species did not aggregate to the 70-100 S species and this led them to consider whether the aggregation of these species constitute a biophysical model for the interaction of the particulate enzyme with the membrane under physiological conditions. Suitable tests of this model would involve examining whether conditions or agents which promote extraction of 14 S and/or 18 S species also prevent aggregation of these species in low ionic-strength media. One such condition which supports the model is treatment with collagenase, which extracts some non-aggregative 16 S enzyme from electric organ (Dudai & Silman, 1974b; Rieger et al, 1973). However, the collagenase extraction also yields much larger amounts of the 11 S species, perhaps by protease contamination, and the action of this agent is complex. Another substance which promotes extraction of some, but not all, of the 14 S and 18 S forms is EDTA in millimolar concentrations (Dudai & Silman, 1973, 1974b), however, the effect of EDTA on aggregation of these species has not been reported.

The converse of this test of the model is to examine whether agents which prevent aggregation of the 14 S and 18 S species also promote their extraction. Several enzymes have been shown to generate non-aggregative species whose sedimentation coefficients differ only slightly from those of the original 14 S and 18 S forms; these include phospholipase C from two microorganisms and a neuraminidase which also affects enzyme-antibody interactions (Rieger

et al, 1973). However, phospholipase C appears not to promote solubilization of the 14 S and 18 S species (Dudai & Silman, 1974b). This failure of the converse of the test does not rule out the model as agents which affect aggregation of the 14 S and 18 S species may not have access to the appropriate sites when the enzyme is membrane bound.

(iii) The enzyme and membrane excitability The proposal that ACh has a general role in controlling ionic currents in excitable membranes which is not limited to its action at synapses has been advocated by Nachmansohn for about 30 years (Nachmansohn & Wilson, 1951). While this basic opinion has not changed, a detailed scheme of cyclic processes which describe this role has evolved over several years (Nachmansohn, 1955, 1959, 1971, 1973) and has recently been formulated as an 'integral model' by Neumann and Nachmansohn (1973). This model is shown in Fig. I.4.

The essential prediction made by this scheme is that ACh, through its sequential interaction with an acetylcholine storage-site S, the acetylcholine receptor R, and the acetylcholinesterase E controls the gate through which ions transiently flow during membrane excitability. The S, R and E are associated in a protein assembly and several such assemblies surrounding an additional gateway structure comprise a basic excitation unit (BEU). Acetylcholine acts to amplify small threshold ionic currents into much larger ionic currents which occur during the action potential. The model is based partially on well-established facts but also includes several detailed proposals which have not yet been tested experimentally. Thus, its hypothetical nature has contributed to controversy about the model.

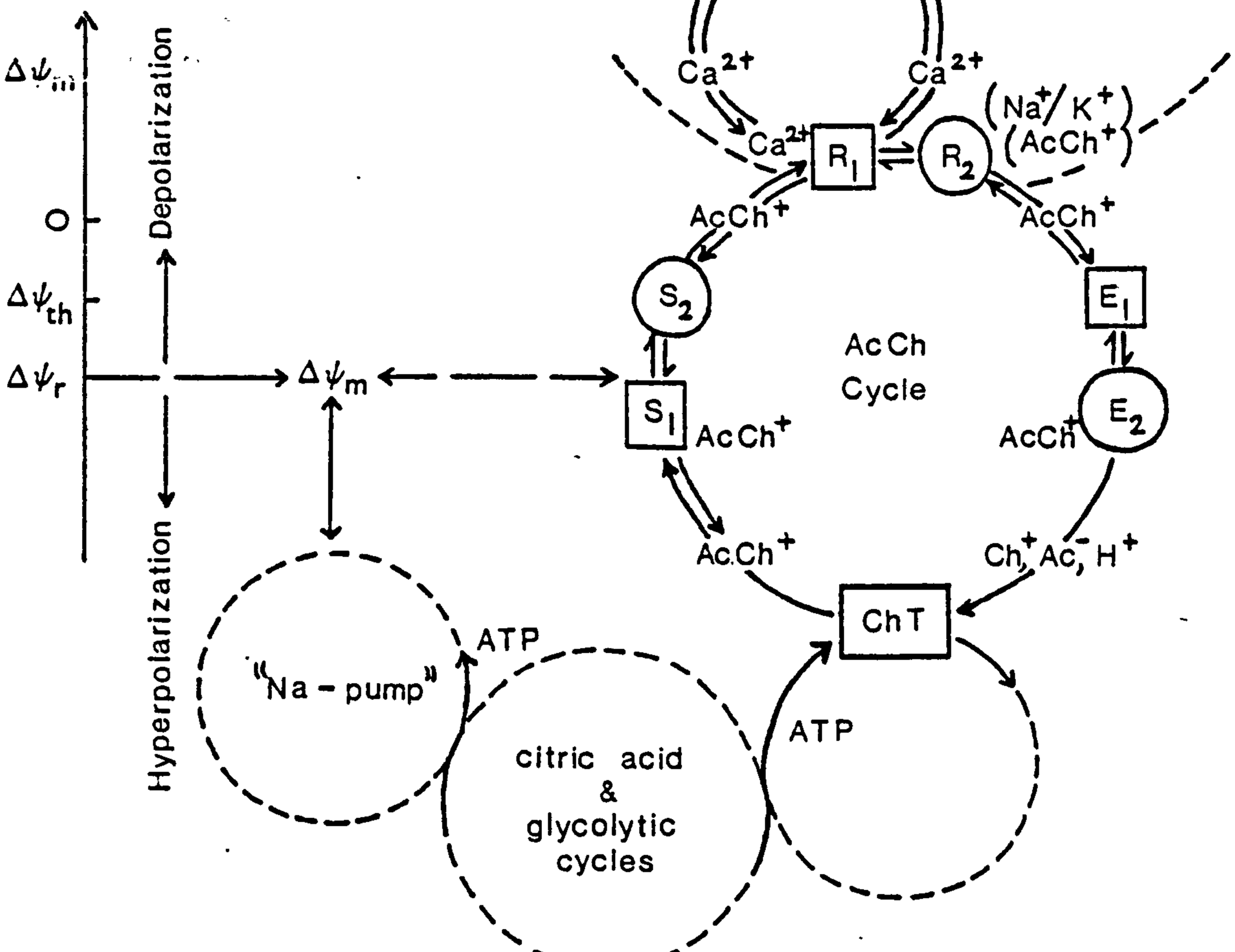


Fig. I.4 Acetylcholine Cycle for the Cyclic Chemical Control of Stationary Membrane Potentials and Transient Potential Changes (Neumann and Nachmansohn, 1975)

The binding capacity of the acetylcholine storage site S is determined by an equilibrium between alternate conformations S_1 and S_2 which is assumed to be dependent on the membrane potential $\Delta\psi_m$. Because $\Delta\psi_m$ is regulated by the "Na^+ pump", the binding capacity is further coupled to the citric acid and glycolytic cycles which interact with the pump. The distribution between open ion gates G_2 and closed gates G_1 is controlled by Ca^{2+} released by the interaction of acetylcholine with the receptor R. Choline-O-acetyltransferase Ch T links acetylcholine ($AcCh^+$)

synthesis with $AcCh^+$ translocation within the assembly of S, R, and acetylcholinesterase, E, and these components define the $AcCh^+$ cycle. The continuous subthreshold flux of $AcCh^+$ through the cycle is maintained by virtually irreversible hydrolysis of $AcCh^+$ to choline (Ch^+), acetate (Ac^-) and protons (H^+) and by a steady supply of acetylcholine to S from the synthesis cycle. In the resting stationary state, the membrane potential ($\Delta\psi_r$) reflects a dynamic balance between active transport (and $AcCh^+$ synthesis) and passive fluxes both of $AcCh^+$ (through the SRE assembly) and of the various ions unsymmetrically distributed across the membrane. Fluctuations in membrane potential (and exchange currents) are postulated to be amplified by coupled fluctuations in the continuous translocation of $AcCh^+$ through the $AcCh^+$ cycle.

This integral model is compatible with a wide variety of structural and functional distinctions between postsynaptic and conducting membrane that have been reported. The ion-gating mechanism included in the model is assumed to differ significantly in these two classes of excitable membrane, as experimental data on the time course of membrane conductance changes (Hodgkin, 1964; Takeuchi & Takeuchi, 1960) and the sensitivity to pharmacological agents like tetrodotoxin (Hille, 1970) show. The density of BEUs and their accessibility to exogenous enzyme inhibitors and receptor agonists and antagonists must also differ between the two membrane classes as reported by Nachmansohn (1971).

In the integral model, the proposed action of endogenous ACh is intracellular; in fact intramembranous. This proposal is in sharp contrast to the intercellular action of ACh presumed by the widely held neurotransmitter theory (Katz, 1969; Hall, 1972). The intramembranous action of the acetylcholine cycle in both synaptic and conducting membranes is predicted by the integral model on the basis of the following arguments which Nachmansohn has offered (Neumann and Nachmansohn, 1975): (1) ACh has never been found outside excitable cells unless eserine is added, that is, unless the powerful biological removal mechanism inherent in AChE is inhibited; thus the appearance of ACh on the outside does not occur under physiological conditions. (2) The action of exogenous ACh and curare at the receptor is usually limited to synapses because most conducting membranes are protected by a variety of structural barriers composed, for example, of myelin or Schwann cells. However, rapid and powerful actions of receptor ligands comparable to those at synapses have been demonstrated in

several appropriate axons and under various experimental conditions. (3) The enzyme and receptor at synapses are present both in nerve terminals and in postsynaptic membranes. ACh can act on both membranes to produce either a postsynaptic cellular action potential or a presynaptic cellular antidromic impulse. The presence and functional activity of enzyme and receptor in the nerve terminal is more readily explained by assuming a similar role of the ACh cycle in both post- and presynaptic membranes than by alternative explanations. (4) Ionic currents in the synaptic gap, contested for a long time, are now well established to accompany nerve activity. They involve a presynaptic efflux of K^+ ions in a concentration which may produce a depolarization of the postsynaptic membrane sufficient to release ACh from its storage sites. This event is then postulated to trigger the postsynaptic cellular action potential. Synaptic junctions are known to release endogenous K^+ following stimulation; and when exogenous K^+ is injected into the synaptic gap, postsynaptic cellular action potentials are produced.

Nachmansohn (1975) had the opinion that these arguments did not disprove an intercellular synaptic role for acetylcholine. Within the hypothetical context of the integral model, the functional distinctions between synaptic and conducting membrane allow the additional postulate of an intercellular action of ACh at the synapse. The role of K^+ as a synaptic transmitter proposed by the integral model is called into question by the failure of K^+ to influence the frequency of miniature endplate potentials (mepps) (Eccles et al, 1942). These mepps are accounted for within the integral model by the spontaneous discharge of BEUs;

the failure of the mepp frequency to be affected by experimental manipulation of the postsynaptic membrane potential (Katz, 1969), in contrast to the significant effects observed during manipulation of the presynaptic potential, has also raised problems for the integral model.

The integral model also predicts that the BEU is a protein complex which includes the enzyme and the receptor. The evidence available about the structural relationship of these components at the synapse does not so far indicate the close physical proximity shown by classical membrane protein complexes in mitochondria and myofibrils. Nevertheless, the enzyme and the receptor are both present as particulate species in conducting membrane; Katz (1969) and Nachmansohn have suggested that the interaction of ACh with accessible receptor in nerve terminals may promote antidromic firing of nerve axons. Possible structural differences between the endplate and nonendplate enzyme (Hall, 1971, 1973) suggest that postulated BEUs in conducting membrane may differ in orientation from those in postsynaptic membrane.

The third prediction of the integral model is that AChE is essential for the maintenance of the action potential in conducting membrane. This has been tested over a period of several years by observing the effects of enzyme inhibitors (Nachmansohn, 1971), particularly phosphorylating and carbamoylating agents which form acyl-enzyme species of varying degrees of reversibility. Such agents block axonal conduction but the inhibitor concentrations at which the block is achieved vary greatly among nerve preparations. For example, nerve axons encapsulated within protective layers of myelin, Schwann cell or similar structural barriers, like an intact

frog sciatic nerve or squid giant axon, require neutral inhibitor concentrations 3 to 5 orders of magnitude greater than those required to block the soluble enzyme. However, axons in which these barriers are minimized, as at Ranvier nodes of single frog sciatic fibres (Dettbarn, 1960), effects are shown with the same inhibitors at much lower concentrations which are comparable to those with which effects are seen at synapses. Specific oxime reactivators of the phosphorylated enzyme were applied to axons in which excitability was blocked, and partial restoration of excitability was demonstrated (Kremzner & Rosenberg, 1971; Nachmansohn, 1971).

The above observations suggest that AChE is essential for axonal conduction but Nachmansohn stated that they do not provide adequate proof. Inhibitors of AChE, particularly acylating agents, may also interact with other cellular components and the proposed involvement of AChE requires a correlation of the enzyme activity with the extent of the conduction block. Some problems arise in establishing this correlation; the first is theoretical and concerns the quantitative relationship of the enzyme activity to conduction; the other two interrelated problems are technical and involve quantitative determination of tissue AChE activities.

Nachmansohn has also emphasized the potential difficulties in assaying enzyme in intact tissue and in homogenates. If AChE is partially located in an internal membrane phase in conducting membrane, the total amount of enzyme assayed would be expected to increase under homogenization conditions in which membranes are solubilized. This is observed for eel electric organ tissue (Dudai & Silman, 1974b) but has not been observed in several

reports on the very low activities associated with axon and muscle membranes (Kremzner & Rosenberg, 1971; Hall, 1973; McIntosh & Plummer, 1973; Liu & Mittag, 1974). The level of active particulate enzyme required for conducting membrane excitability could amount to only a few per cent of the total enzyme in such a tissue. Owing to the difficulties associated with establishing a correlation between enzyme activity and membrane conduction, the question of the general role of acetylcholinesterase in excitable membranes may not be resolved until tissue culture mutants entirely lacking the enzyme are available.

2. MEMBRANE-BOUND ENZYMES

A. Cell Membranes

(i) General properties Generally, it has been recognized that cell membranes are the site of most vital functions, such as energy supply, active transport, neural function, vision, excitation-contraction coupling, and photosynthesis. In addition, recent studies have shown the importance of structural and organizational aspects of membranes for the chemical reactions taking place in living cells.

A most significant development in the field of membranes has been that of a conceptual change. At the turn of the century, cell membranes were considered mainly as passive barriers in which lipids were essential in preventing easy passage of cell constituents and metabolites. Presently, it is well established that cell membranes contain many proteins including enzymes and in some membranes 30-50 different proteins have been isolated and identified. Biomembranes have been recognized as extremely dynamic structures and also the site of numerous chemical processes.

In many biological membranes, a greater proportion of the mass are represented by proteins (more than two-thirds) and the rest by phospholipids (about one-third). In a given type of membrane, the phospholipids and their chemical properties are diverse, and the diversity increases when different membrane types are compared. There are also oligosaccharides and various small molecules, including metal ions present in the membranes. However, the specificity, the diversity, and the efficiency of membrane functions

seem to be more readily accounted for when the dominant role is attributed to the membrane proteins rather than to the lipid phase.

Even though there is a large amount of information on the chemical composition of membranes, the real knowledge of the molecular organization of the various constituents in the intact membrane is still uncertain. The notion of 'unit membrane', proposed by Robertson (1960) and based essentially on the Danielli-Davson model, assumed a uniform structure of all cell membranes 80 Å thick and formed by a bimolecular leaflet of phospholipids to which proteins are attached on the inside and outside by ionic forces. This notion first appeared attractive to many investigators but it proved to be inadequate to account for the great variety of biochemical, biophysical, and electron microscopic examinations (Sjöstrand, 1963; Elbers, 1964; Green & Perdue, 1966; Korn, 1966; Sjöstrand & Barajas, 1968; Green & McLennan, 1969). Singer (1971) has discussed various thermodynamic problems due to the complexity of membrane structure and he has pointed out the difficulties of reconciling the Robertson model with established thermodynamic facts.

In recent years various membrane models have been proposed in an effort to integrate the available information (Lenard & Singer, 1966; Benson, 1968; Blasie & Worthington, 1969; Vanderkooi & Green, 1971; Singer, 1971). Some of these membrane models will be discussed to illustrate the remarkable development of the last decade. The model proposed by Sjöstrand and Barajas (1970) for the inner mitochondrial membrane incorporates the enzymes and coenzymes with their subunits biochemically established to be

located in this membrane. It also accounts for the proper relationships between the amounts of proteins and phospholipids in this particular membrane. This model is rather attractive because it integrates the great number of known constituents. Sjöstrand and Barajas have introduced new techniques for the preparation and fixation of the specimen for electron microscopy, avoiding the usual standard techniques which almost certainly denature the proteins. Using these techniques they found that membranes have a thickness of 150-200 Å as compared to the 80-100 Å obtained with the standard procedures and their electron micrographs also show many globular formations within the membrane (Sjöstrand & Barajas, 1968).

The 'unit membrane' hypothesis has now been replaced by a more feasible model introduced by Singer and Nicolson (1972) known as the 'fluid mosaic model'. This model explains many physical properties of membranes and it assumes that the phospholipids form a bilayer with the proteins embedded in the lipid. In addition, most molecules are assumed to be free to move in the plane of the membrane.

(ii) Membrane protein classification . It has been suggested that the proteins associated with membranes can be generally classified into two categories termed peripheral (Singer & Nicolson, 1972) (extrinsic [Capaldi & Green, 1972] or membrane-associated [Fleischer et al, 1971]) and integral (Singer & Nicolson, 1972) (intrinsic [Capaldi & Green, 1972]). This classification is operational and based on the manner these proteins can be detached from the membrane (Singer, 1974). The peripheral proteins can be

relatively easily dislodged, usually in lipid-free form, from membranes by chelating agents, by decreasing or increasing the ionic strength or the pH without dissociating the lipid matrix of the membrane. These proteins are usually water-soluble after extraction, and include proteins like cytochrome c, the tertiary structure of which is known and is fairly similar to those of other water-soluble globular proteins having a hydrophilic surface and hydrophobic interior (Dickerson, 1971). These proteins are assumed to be bound by predominantly polar interactions to the proteins or the lipids in the membrane (Jacobs & Sanadi, 1960).

The integral proteins are tightly bound to the membrane and can only be solubilized by disrupting the membrane with organic solvents or by detergents. These proteins have been suggested to have an amphiphilic structure (Lenard & Singer, 1966; Wallach & Zahler, 1966). Evidence supporting this view is beginning to appear. Cytochrome b_5 of the endoplasmic reticulum has a hydrophilic globular part (Mathews et al, 1971) with a molecular weight of 11,000 which is anchored to the lipid matrix by a 5,000 dalton peptide segment enriched in apolar amino acids (Ito & Sato, 1968; Spatz & Strittmatter, 1971).

The suggestion that the lipid bilayer was covered on both faces with protein was put forward as a result of the observation of the low surface tension of cells, compared to lipids (Danielli & Davson, 1935). Electron microscopy of membranes showed their dimensions to be in the region of 60-75 Å, whereas lipid bilayers have dimensions of 40-45 Å. The apparent difference due to protein was about 30 Å, and therefore, most of the protein was suggested to be present in the form of an extended monolayer or β-structure

(Robertson, 1964). However, recent spectroscopic studies (Wallach & Zahler, 1966; Lenard & Singer, 1966; Glaser & Singer, 1971; Urry, 1972; Zahler et al, 1972) of various membranes have shown a considerable amount of α-helical character and less β-structure, suggesting that much of the membrane protein may be globular rather than an extended sheet. In addition, recent X-ray diffraction analyses of several membranes have shown that the membrane dimensions are appreciably greater than those observed by electron microscopy and are in the region of 100-120 Å (Finean et al, 1968), which would allow for the presence of globular proteins. Although the proportions of catalytic protein to total protein cannot be determined, it is significant in this respect that even the so-called 'structural protein' of membranes has been shown to contain a considerable amount of denatured catalytic protein (Senior & McLennan, 1970). The high α-helix content in membrane proteins should cause little surprise since soluble enzymes have long been shown to be globular proteins. Variation in the protein content of membranes is great, ranging from approximately 20% (myelin) to 75% (mitochondrial inner membrane) (Guidotti, 1972).

(iii) Lipid-protein associations At neutral pH, most of the membrane phospholipids (phosphatidylcholine, phosphatidylethanolamine, sphingomyelin) are zwitterionic; and the minor lipids (phosphatidylserine, phosphatidylinositol, cardiolipin, phosphatidylglycerol) bear a net negative charge. The head-groups of the membrane phospholipids are available for electrostatic bonding and this may be a feature of membrane structure.

X-ray diffraction studies have shown separate lipid and lipoprotein phases, upon drying erythrocyte ghosts. Some experiments

have indicated that the lipids which separated out under these conditions were mainly the zwitterionic lipids, whereas those associated with the protein were largely the more polar lipids, suggesting ionic interactions between membrane proteins and these lipids (Finean & Coleman, 1970).

The lipid requirement for some enzymes shows a high degree of specificity which reflects a very precise role for the particular lipid in the conformation of the enzyme protein, involving the polar head-groups of the lipid. Such specific reactions may explain the presence of the small quantities of these highly polar lipids in membranes. However, some experiments suggest that a proportion of the phospholipid head-groups may not be interacting strongly with protein (Vanderkooi & Martonosi, 1969; Coleman et al, 1970; Glaser et al, 1970). The lipids which were shown to have polar head-groups that did not interact strongly with proteins and thus were available for phospholipase attack included phosphatidylcholine and sphingomyelin, and a proportion of phosphatidylethanolamine.

As mentioned earlier, some membrane proteins are probably bound to membranes only by electrostatic interactions, in that they can be dissociated by salt solutions or chelating agents. The remaining proteins (integral, intrinsic or membrane-associated) must be held by other forms of bonding, in addition to the purely electrostatic. Effective use of detergents and solvents such as n-butanol in the solubilization of membrane proteins suggests the involvement of hydrophobic interactions between membrane proteins and lipids. These hydrophobic associations have been demonstrated by spectroscopic studies (ORD, CD, NMR) and have been attributed

mainly to protein-lipid chain interaction, though some protein-protein interaction is also possible (Singer, 1971; Wallach & Zahler, 1966; Lenard & Singer, 1966; Chapman et al, 1968).

The amino acid composition of membrane proteins has not revealed the predominance of polar residues, which would have been expected for a membrane structure in which proteins were only associated electrostatically with lipid in the β-configuration. However, these analyses have shown a slight excess of non-polar residues in membrane proteins from various sources and recent data comparing purified 'intrinsic', 'extrinsic' and soluble proteins has shown that many of the intrinsic proteins possess more non-polar character than the other proteins (Capaldi & Vanderkooi, 1972).

B. Features of Enzymes Resulting From the Association with Membranes

(i) Localization of reaction

(a) Compartmentation In free solution, the rate of enzyme reaction is partly dependent upon the number of successful collisions between substrate and enzyme in unit time. The number of collisions is dependent upon the concentration of substrate and enzyme, and in free solution, the concentration of substrate or enzyme beyond certain limits is not feasible on either osmotic or energy-economy grounds. If the enzyme were restricted to an intracellular compartment whose boundary would not allow passage of the substrate, then a situation is created in which relatively high local concentrations of substrates could be maintained. In addition to the overall economy which compartmentation brings, there

are implications with respect to competition (physical separation of competing processes), metabolic control (the overall rate of a metabolic pathway may be determined by the rate of penetration of the substrate into the compartment) and the polarity of the cell (localization of some enzyme-bearing membranes at one pole only).

(b) Multienzyme sequences Incorporation of the two enzymes which are linked by a common metabolite, locally into the same membrane would reduce the diffusion distance of the substrate to the enzyme. Therefore, it is not unusual to find many membranes bearing examples of multienzyme sequences, in which the product of one enzyme becomes the substrate for the next enzyme in the sequence. Such close proximity of enzymes is apparent especially where highly unstable (usually free radical) intermediates are produced (e.g. in some redox reactions).

(c) Lipid phase Lipids are present in all membranes. The lipid may have an important role in the function of some membrane enzymes, by providing an alternative non-aqueous phase in which some reactions might occur. Lipid metabolism must take place either in a lipid medium or at the lipid-water interface and hence one of the functions of the membrane may be to provide a hydrophobic medium in which lipid metabolism may occur.

(ii) Microenvironment Allotopy (Racker, 1967) occurs in several enzymes. This phenomenon was first described for mitochondrial ATPase (F_1). In its membrane-bound form the enzyme was sensitive to oligomycin and N,N-dicyclohexylcarbodiimide but in its soluble form, this sensitivity was lost and the enzyme also became cold labile. The specific properties differing between

soluble and membrane-bound forms vary from one enzyme to another, but amongst those so far reported are: (i) inhibitor sensitivity (Racker, 1967; Singer & Gutman, 1971; Cerletti & Zanetti, 1971; Fessenden-Raden & Racker, 1971; Razin, 1972), (ii) cold lability (Racker, 1967; Razin, 1972), (iii) pH optimum (Silman & Karlin, 1967; Katchalski et al, 1971), (iv) K_m (Katchalski et al, 1971), (v) redox potential (Singer & Gutman, 1971; Cerletti & Zanetti, 1971), (vi) stability (Coleman & Hubscher, 1963; Cerletti & Zanetti, 1971), (vii) reconstitutive ability (Racker, 1970; Fessenden-Raden & Racker, 1971; Razin, 1972), (viii) substrate specificity (Rossi & Cariagni, 1971).

A membrane-bound enzyme exists in a characteristic micro-environment due to the effects of the membrane on which it is located. As a result its properties may be influenced by: (i) the general chemical and physical nature of the membrane (composition, lipophilic nature, charge, dielectric constant), (ii) by specific interactions with individual molecules in its immediate neighbourhood (proteins and lipids), (iii) by the local effects of its own action and that of its neighbours. In addition, alterations in the properties of the membrane by changes in its general composition, molecular arrangement or specific components may be important regulatory influences on some membrane-bound enzymes.

(iii) Problems in assay In addition to the normal difficulties encountered in the assay of a soluble enzyme, membrane-bound enzymes also give rise to assay problems of their own. These problems arise because these enzymes are particulate, they are influenced by the microenvironment, and they are spatially organized.

(a) Particulate nature Owing to the particulate nature of membrane-bound enzymes, sedimentation may occur during assay. In the sediment, inward diffusion of substrate may be limiting, local product accumulation may take place, and possibly local pH changes. These factors will influence the kinetics of the assay and to ensure homogeneous conditions it is necessary to agitate the assay mixture.

(b) Microenvironmental influences Changes in the membrane in which the enzyme is located, prior to or during the assay may have considerable effect on the enzyme activity (Bramley et al, 1971; Hanahan & Ekholm, 1972). Since the membrane may also contain many other enzymic activities than the one being assayed, and purification may not always be practical, a careful choice of assay and the use of inhibitors for other competing enzymes are necessary.

(c) Spatial organization The spatial relationship between the enzyme and the membrane may mean that the enzyme is located on the outer face, on the inner face, or even spanning the membrane. The membrane may (and often does) have limited permeability for the substrate and so the morphological and physiological condition of the membrane will have a marked effect upon the assay. If the membranes are in the form of open sheets or if they have been solubilized, then permeability limitations will not apply. However, if the membranes are in the form of intact organelles or closed vesicles, permeability limitations will be evident when the active centre of the enzyme is located in the inside of such structures. Stimulation of the appropriate carrier,

substitution of a more lipid-soluble substrate or destruction of the permeability barrier (by homogenization, sonication, detergent action, hypotonic or alkaline conditions) (Gianetto & De Duve, 1955; Lin, 1971) ensure that the substrate is presented to the active site. In some cases a delicate balance must be maintained between activation by improving permeability and inactivation of the enzyme by the detergent (Bramley et al, 1971). It is also possible that preparations of apparently the same membranous material may have differing permeabilities, depending upon the conditions of preparation, suspending solutions, and osmolarity (Bramley et al, 1971; Hanahan & Ekholm, 1972) and this will introduce further difficulties in the assay.

The phenomenon of 'membrane inversion' may also complicate some observations. Under such conditions, the normal membrane situation of outside-out is reversed, and the inside face is presented to the suspending medium (Wallach & Kamat, 1964; Steck et al, 1970; Wallach, 1972). This has implications for latency and the exposure of normally internalized activities (Racker, 1970). The assay of enzymes may also be complicated when the enzyme activity spans the membrane (Whittam, 1962; Stetton & Burnett, 1967).

C. Properties of Detergents in Aqueous Solution

(i) Detergents - a special group of lipids Generally, lipids form a heterogeneous group of molecules which include hydrocarbons, pigments, cholesterol, phospholipids, glycolipids and detergents. Apolar groups of aliphatic or aromatic nature are present in all lipids and most lipids have polar groups. The aromatic and aliphatic moieties are hydrophobic and the low solubility of apolar

groups in aqueous solution is mainly due to the strong interactions between water molecules (Hartley, 1936; Frank & Evans, 1945; Kauzmann, 1959; Tanford, 1973). Little compensating attraction occurs between water and the solute, for a hydrocarbon, and the water molecules closest to the solute are forced into ordered cage-like structures. Also, the internal mobility of hydrocarbon chains is reduced (Aranow, 1963), resulting in loss of entropy and is energetically unfavourable.

The polar groups may be charged such as phosphate, amino, sulphate and carboxyl groups, or neutral such as hydroxyl, carbonyl, ester or protonated carboxyl groups. These are hydrophilic, forming strong noncovalent bonds with the surrounding water which are more than sufficient to compensate for the loss of hydrogen bonds between the water molecules. The non-ionic groups are weaker hydrophiles than the charged groups as their energy of interaction with water is lower (Tanford, 1973). In alkyl chains, the hydrophilic groups in lipids are often called heads and the hydrophobic groups tails. Molecules which are partly hydrophilic and partly hydrophobic are called amphiphiles.

The balance between hydrophilic and hydrophobic moieties differ greatly in different lipids and this is reflected in their behaviour in water, thus providing a basis for their classification (Small, 1970). Soluble amphiphiles, which include the detergents used for membrane solubilization, differ from the insoluble and the swelling amphiphiles (which constitute the major lipid groups in biological membranes) essentially only in having a more hydrophilic character.

The soluble amphiphiles can be sub-divided into two groups,

types A and B. Type A includes all those amphiphiles which can form liquid crystals at high concentrations. The hydrophobic moieties of the type A amphiphiles are aliphatic or arylaliphatic. Type B includes those amphiphiles which do not display lyotropic mesomorphism (forming liquid crystals at high concentrations), presumably because of their bulky and complicated cyclic or aromatic hydrophobic moieties. Soluble amphiphiles are also referred to as surfactants and detergents. The term surfactant is used synonymously with soluble amphiphile, whereas the term detergent will be restricted to those soluble amphiphiles that effectively bring about the solubilization of membrane lipids.

(ii) Detergents used in membrane studies In general, commercial surfactants are chemically impure. They may contain varying amounts of water and additives; one batch may differ from the next; and after prolonged storage of liquid non-ionic surfactants, the composition at the bottom of the container may differ from that on the top. Thus, it is advisable to purify the surfactants used whenever possible, and in the case of non-ionic surfactants, which are difficult to purify (Shinoda et al, 1963), the degree of purity of the surfactant used should be determined (Schick, 1967). Ionic surfactants such as bile salts can usually be purified by crystallization. It should be noted that even trace impurities can sometimes be a nuisance in biochemical investigations and so it is necessary to eliminate them whenever possible.

The heterogeneity of non-ionic surfactants also poses another problem. Unless fractionated these have polydisperse polyoxyethylene head-groups due to the purely statistical polymerization of ethylene oxide (Shachat & Greenwald, 1967). In addition, there is

heterogeneity in the hydrophobic part of synthetic surfactants since inhomogeneous fatty acids and alcohols are usually used in their synthesis (Schick, 1967; Makino et al, 1973).

Griffin (1949) has introduced an arbitrary empirical quantity called the hydrophile-lipophile balance (HLB) so that a measure for the balance of size and strength of the opposing hydrophilic and hydrophobic groups in non-ionic surfactants can be obtained. The most hydrophobic materials have a low HLB value (1-10) and increasing HLB value corresponds to increasing hydrophilic character. The HLB value helps to predict the usefulness of surfactants for particular applications and the membrane solubilizing power of non-ionic detergents depends on their HLB value. (see Table I.4).

D. Effects of Detergents on Biological Membranes

(i) Solubilization of membranes There are no generally accepted criteria for membrane solubilization but in pure lipid systems, solubilization can be defined as the formation of mixed micelles. However, the more complex nature of the membrane solubilization process does not permit such a simple definition (Penefsky & Tzagoloff, 1971; Razin, 1972; Kagawa, 1972; Wallach & Winzler, 1974). The criteria used are operational, and are usually based on the decrease in turbidity of the membrane solution, the increase in non-sedimentable material and the disappearance of continuous lamellar membranes as seen in electron microscopy.

It has been shown that primarily the monomer form of the detergent is bound to the membrane and not the micellar form (Tanford, 1972). However, binding of micelles to membranes as an alternative binding mechanism when membranes and micellar solutions are mixed

Table I.4

Surfactant	Commercial Name	HLB-number
PEG (10) stearyl alcohol	Brij 76	12.4
PEG (9-10) p-t-octylphenol	Triton X-100	13.5
PEG (20) sorbitol monostearate	Tween 60	14.9
PEG (20) sorbitol monopalmitate	Tween 40	15.6
PEG (20) sorbitan monolaurate	Tween 20	16.7

PEG : polyoxyethyleneglycol

cannot be completely ignored (Auborn et al, 1971; Choules et al, 1973). There is also evidence indicating that bile salts may begin to bind to membranes first at the CMC (critical micellar concentration) or just below it (Jörgensen & Skou, 1969; Phillipot & Authier, 1972). Bile salts cause lysis, however, at concentrations far below the CMC (Roelofsen et al, 1971; Kreibich et al, 1973) which suggests that at least some binding must occur at these concentrations.

The free equilibrium concentrations of sodium dodecylsulphate and Triton X-100 have been shown to remain below or equal to the CMC in the presence of membranes, the excess being bound to the membrane or to components released from the membrane (Helenius & Söderlund, 1973). The binding of detergent monomers to membranes prevents the formation of pure detergent micelles by reducing the free detergent concentration below the CMC. Therefore micelles of these detergents do not occur together with membranes in solution unless the detergent is present in a large excess, which completely solubilizes the membrane (Tanford, 1972). In the case of surfactants that form small micelles (e.g. cholate), the monomer concentration keeps rising significantly at concentrations above the CMC. In these cases membranes and micelles may in theory occur together at concentrations above the CMC although actual experimental evidence is lacking.

The degree of solubilization depends mainly on the amount of detergent bound and can therefore best be correlated with the ratio of bound detergent to membrane. This ratio is often rather laborious to determine, however, it is a common experience that the effects of detergents correlate quite well also with the total

detergent/membrane ratio in the sample, especially if the concentration of membranes is relatively high. Under such conditions, the free unbound detergent will constitute just a small portion of the total detergent in the sample. The lower the CMC of the detergent and the higher the concentration of the membrane the closer will the total detergent/membrane ratio be to the bound detergent/membrane ratio.

With sodium dodecylsulphate and Triton X-100, the ratio of detergent to membrane lipid corresponds roughly to that needed for phase transition in phospholipids, when determining the amounts of detergent needed to solubilize various isolated biological membranes (Helenius & Simons, 1975). The amount of deoxycholate required to solubilize the membranes varies from one report to the other, more than for the other detergents, which may partly reflect the well-documented dependence of bile salt effectiveness on the presence of salts and the pH (Tzagoloff & Penefsky, 1971).

The detergents that have been indicated as effective membrane solubilizing agents are probably the ones which have high affinity for the membrane compared to their tendency to form micelles, and in addition, have molecular properties that effectively bring about the dissociation of membranes when bound.

For non-ionic surfactants there is some correlation between structure and solubilizing potency. Studies with mitochondria, microsomes, viral membranes and bacterial membranes show that most of the effective surfactants are in the 12.5-14.5 HLB range. Those with higher HLB values (e.g. Tweens) are applied in membrane studies. They release material (mainly peripheral protein) from many membranes but do not dissociate the lamellar membrane

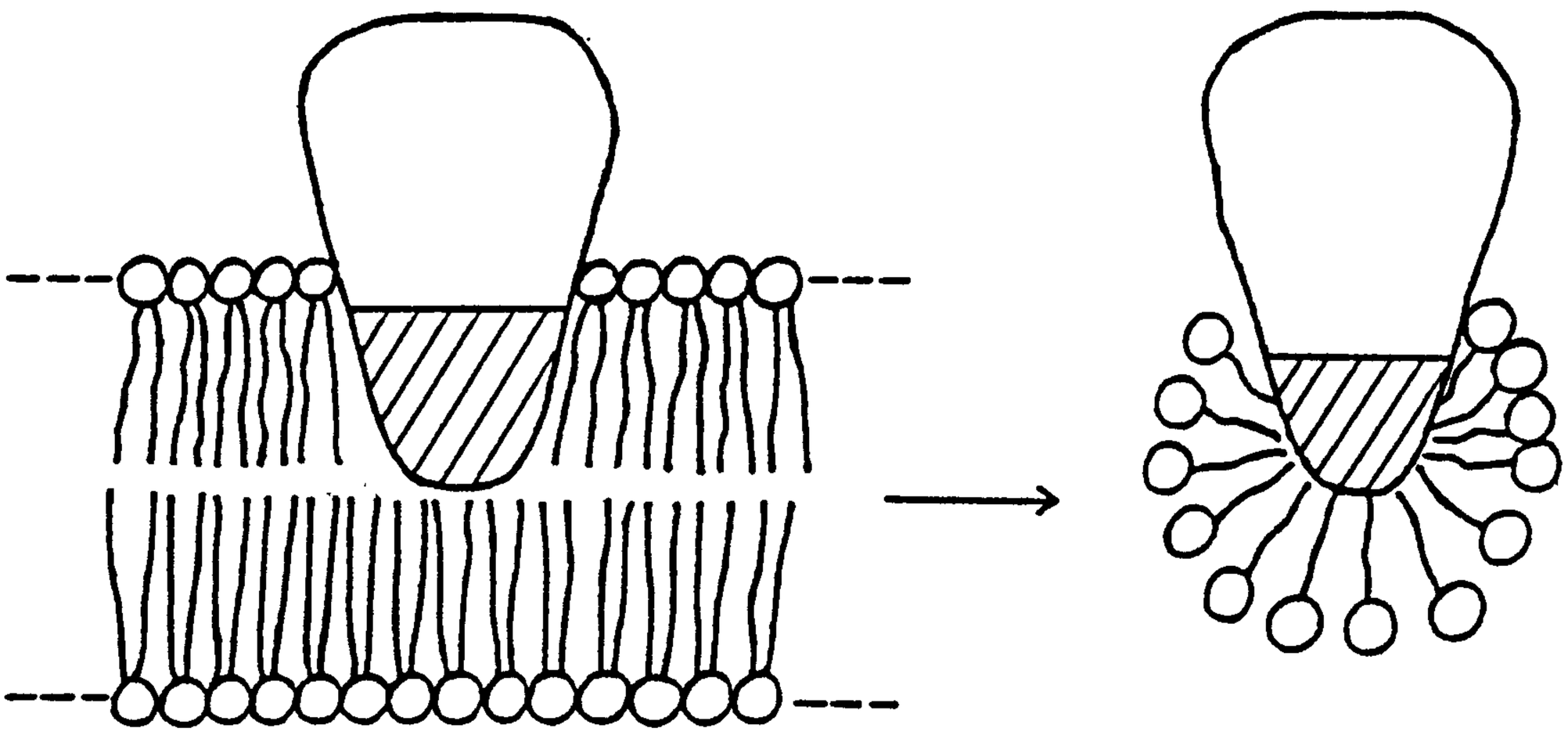
structure at the concentrations normally used for solubilization (<5%). The lipids and the integral proteins are released only if the extraction is done at very high pH (Hosaka, 1968) or if it is repeated several times (Zorn & Futterman, 1973; Hjertén & Johansson, 1972).

The anionic and cationic detergents of type A such as sodium dodecylsulphate, not only displace the lipids from those membrane proteins that bind to lipids but due to the high detergent monomer concentration needed dor delipidation, the cooperative mode of binding to the membrane proteins cannot usually be avoided. Sodium dodecylsulphate binds to both peripheral and integral proteins which thereby usually undergo drastic conformational changes and loss of biological activity.

In contrast to the denaturing detergents, Triton X-100 and deoxycholate appear to interact mainly with those proteins which are bound to the membrane lipids by hydrophobic interactions. In most cases, the binding of these detergents does not lead to major conformational changes of the protein and loss of activity. The action of these mild detergents is shown in Fig. I.5. Helenius & Simons (1975) have postulated that the detergent molecules are bound to the hydrophobic part and not to the hydrophilic parts of the amphiphilic protein. More experimental evidence is required to decide whether the detergent molecules are all bound to individual sites or whether only a part of the bound molecules would interact directly with the protein and the rest bind cooperatively to form a micelle-like region on the protein surface; a micelle-like interaction appears more likely (Robinson et al, 1974).

Although Triton X-100 and the bile salts do not usually

Fig. I.5 Hypothetical Mechanism for the Two-phase Extraction (delipidation) of an Amphiphilic Membrane Protein (Helenius and Simons, 1975)



In the soluble detergent-protein complex seen to the right, the bound detergent molecules form a micelle-like region around the hydrophobic domain of the protein (shaded). The detergent does not interact with the hydrophilic parts of the protein.

denature protein, there are some membrane proteins which lose their biological activities when solubilized with these detergents, for example, $(Na^+ + K^+)$ - ATP ase, Ca^{2+} - ATP ase, cytochrome oxidase, glucose-6-phosphatase, and hormone responsiveness of adenylate cyclase. This may be due to (1) the proteins being less resistant to denaturation (the constraints that hold the proteins in their active conformation may be affected by the disruption of essential protein-lipid or protein-protein interactions during solubilization), (2) detergent binding to the functional sites on the proteins, (3) removal of cofactors necessary for activity, (4) to inactivation because of assay problems. For example, several membrane enzymes have been found to depend on specific lipids for activity (Rothfield & Romeo, 1971; Coleman, 1973) and when these lipids are removed, the enzyme is inactivated. Many of these studies have used fairly harsh procedures for delipidation (organic solvent extraction, impure phospholipases) and in some cases, the requirements obtained may depend more on the life history of the protein than on specificity. Different groups working with the same membrane protein (e.g. $(Na^+ + K^+)$ - ATP ase [Roelofsen & van Deenen, 1973], rhodopsin [Zorn & Futterman, 1971; Hong & Hubbel, 1973]) have come to different conclusions regarding their lipid specificities.

Among the currently available procedures, Triton X-100 (and probably other non-ionic detergents) appears to provide the most generally applicable solubilization agent to extract and purify an integrally bound membrane protein lipid-free in its native conformation or something close to it. Triton X-100 also appears to be more gentle in its effects on proteins than the bile salts

(Soltysiak & Kaniuga, 1970: McFarland & Inesi, 1971; Meyer, 1971; Kirkpatrick & Sandberg, 1973) but there are examples for which the reverse is true (Hatefi, 1966).

(ii) Effects on protein-protein interactions Many membrane proteins possess quaternary structure and the non-covalent interactions between membrane proteins may be permanent or reversible depending on their functions in the membrane (Capaldi & Green, 1972; Singer, 1974; Steck, 1974). Mild detergents are usually unable to dissociate the protein-protein interactions which keep oligomeric structures together and so they are solubilized, delipidated and kept in solution as units. Whether all the peptides in the oligomeric complex are essential for biological activity is usually uncertain, and neither can one be completely sure that artefactual association of polypeptides does not occur.

The junctions connecting plasma membranes of neighbouring cells in animal tissue are resistant to the action of deoxycholate, non-ionic detergents and lauryl sarcosinate, and are probably characterized by strong non-covalent protein-protein interactions (Singer, 1974). Such protein-protein interactions are also indicated in synaptic membranes (De Robertis et al, 1967; Cotman et al, 1971), erythrocyte membranes (Yu et al, 1973), mitochondria (Hall & Crane, 1972) and the outer membrane layers of the Gram-negative bacteria. The inefficiency of non-ionic surfactants in solubilizing the membrane may be due either to inability to disrupt and penetrate the outer protein coat (especially in animal membrane viruses) or to dissociate the electrostatic bonds between lipid and protein.

The conditions which are known to facilitate the dissociation

of proteins, such as high pH. (Miller, 1970; Tzagoloff & Penefsky, 1971; Umbreit & Strominger, 1973), low (Miller, 1970) and high (Tzagoloff & Penefsky, 1971) ionic strength, metal chelators (Schnaitman, 1971) and urea (Loach et al, 1970; Suginaka et al, 1972), generally also increase the solubilization efficiency of detergents. These conditions may change the properties of the surfactant as well, and consequently the CMC and micellar properties may alter. By increasing the counter ion concentration, the CMC is reduced, and this may partly explain the increased solubilization by ionic detergents with increasing salt. Therefore, bile salts are usually used in solubilization procedures in the presence of high concentrations of sodium chloride or potassium chloride (Tzagoloff & Penefsky, 1971). Although there is no obvious explanation, sucrose has in some cases been observed to increase the efficiency of non-ionic detergents (Thompson & Bachelard, 1970).

3. ISOLATION OF ACETYLCHOLINESTERASE

A. Solubilization

The presence of a heterogeneous size distribution of AChE in soluble extracts from nearly every nerve, muscle and electric organ tissue source which has been examined suggests that the properties of the isolated enzyme may reflect the solubilization procedure. This suggestion has been supported and confirmed by Dudai & Silman (1973) and Hall (1973) who showed that species of lower sedimentation coefficient are more readily solubilized from eel electric organ and rat diaphragm, respectively. In addition, the alterations in sedimentation coefficients of AChE species from eel or torpedo electric organ by added proteases can occur either before or after solubilization of the enzyme (Massoulié & Rieger, 1969; Dudai et al, 1972a; Taylor et al, 1974). It has also been observed that similar or perhaps more drastic alterations may arise from endogenous proteases being exposed to the enzyme during tissue storage or after homogenization (Dudai et al, 1972a; Rieger et al, 1972a). Incubation of eel electric organ extracts with eel spinal cord extracts resulted in the conversion of the 14 S and 18 S enzyme species not only to the 11 S but also to a globular 7.7 S species (Rieger et al, 1972b) whose size corresponded to that of a major component of purified mammalian brain AChE (Chan et al, 1972a).

Two solubilization procedures which lead to well-defined AChE populations have been developed for the eel or torpedo electric organ tissue. One procedure exposes the enzyme to autolytic or proteolytic agents for a time sufficient to produce an extract containing only 11 S activity. Fresh tissue homogenates, when treated with trypsin, yield 11 S AChE, although the required trypsin concentration and time

of exposure vary significantly among the eel and the torpedo enzymes (Massoulié & Rieger, 1969; Dudai et al, 1972a; Taylor et al, 1974). Chen et al (1974) and Dudai et al (1972a) stored eel tissue slices in toluene at 4°C for 1 to 2 years and they obtained extracts which had only 11 S activity. Rothenberg and Nachmansohn (1947) initiated the procedure of immersion of diced eel electric organ tissue in toluene at 4°C for 1-month periods. They found that this method facilitated subsequent extraction and purification procedures while maintaining significant amounts of total AChE activity. Nachmansohn used this procedure to store large quantities of tissue slices prior to large-scale extraction of the enzyme and this method also prevented bacterial contamination. Dudai et al (1972a) and Chen et al (1974) reported that the rate at which the enzyme was converted to the 11 S form during tissue storage in toluene was partial after 2 months and complete after 2 years.

Another solubilization procedure for eel enzyme involves homogenization of fresh or freshly frozen electric organ tissue in 1 mol/l NaCl and this was suggested by the observation that membrane-bound acetylcholinesterase is solubilized in 1 mol/l NaCl (Silman & Karlin, 1967). Comparison of this extraction procedure with a parallel procedure, involving partial autolysis by immersion of tissue slices in toluene at 4°C for relatively short periods led Massoulié and Rieger to their description of the 8 S, 14 S and 18 S forms in fresh high ionic-strength extracts and also of the conversion of these species to the 11 S form (Massoulié & Rieger, 1969). Homogenization in buffered 1 mol/l NaCl has been used as the solubilization procedure prior to purification of fresh electric tissue species (Dudai et al, 1972; Rieger et al, 1973a; Chen et al, 1973) and this procedure appears to be efficient for the solubilization of

these species at the present time. Dudai & Silman (1974b) have demonstrated that the addition of 0.5% Triton X-100 to high ionic-strength buffer increased the AChE which could be extracted from fresh eel tissue by up to a factor of 2, without significant alteration of the species distribution. Reichard et al (1973) have also reported that AChE may be extracted by treatment of lyophilized electric organ tissue with n-butanol. Until about 1970 the extraction procedure most frequently used for electric eel tissue enzyme involved storage in toluene for a period of weeks followed by homogenization and fractionation in high ionic-strength ammonium sulphate (Rothenberg & Nachmansohn, 1947; Lawler, 1959; Kremzner & Wilson, 1963; Hargreaves et al, 1963). This procedure extracts a mixture of the 11 S with the 14 S and 18 S species and since the time of immersion in toluene generally was not carefully controlled or even specified, this mixture was obtained in varying proportions from one preparation to another and led to conflicting reports concerning the properties of AChE (Lawler, 1963; Kremzner & Wilson, 1964; Millar & Grafius, 1965), suggesting that an appropriate extraction procedure is thus essential to a precise characterization of the solubilized enzyme.

Preferential solubilization of certain AChE species at low ionic-strength also revealed the importance of the solubilization procedure. Homogenization of eel electric organ at low ionic-strength resulted in partial solubilization of both aggregative and nonaggregative species but when 10 mmol/l $CaCl_2$ was added, only the nonaggregative 8 S and 11 S species were solubilized (Dudai & Silman, 1973). Burger et al (1968) demonstrated that Ca^{2+} retarded the solubilization of AChE from mammalian erythrocytes and conversely, Dudai and Silman (1974b) showed that 2.5 - 5.0 mmol/l EDTA resulted in an increased solubilization of the eel enzyme at low ionic-strength, presumably

by complexing endogenous Ca^{2+} and allowing solubilization of some aggregative species. However, the increased solubilization obtained with EDTA is only about one-third that obtained with 1 mol/l NaCl and one-sixth that with 1 mol/l NaCl and 0.5% Triton.

In some mammalian systems, detergent appears essential for the solubilization of particulate AChE. The combination of high salt concentrations and 0.5 - 1.0% Triton X-100 resulted in solubilization of the membrane-bound enzyme from human erythrocytes (Wright & Plummer, 1972), rat diaphragm (Hall, 1973), chick muscle (Dudai & Silman, 1974b), and pig brain (McIntosh & Plummer, 1973), while the use of 1 mol/l NaCl alone gave little or no solubilization above that seen in low ionic-strength buffers. Soluble (naturally soluble) enzyme species from some of these sources have been extracted with low ionic-strength buffers and the enzyme species obtained had lower sedimentation coefficients and/or appeared smaller on gel-exclusion columns than membrane-bound enzyme species (Hall, 1973; McIntosh & Plummer, 1973; Rieger et al, 1972b). Chan et al (1972a) has also demonstrated that EDTA (1-2 mmol/l) could double the soluble enzyme extracted by low ionic-strength buffer from bovine brain caudate nuclei; whether this increase reflected the solubilization of membrane-bound species was not investigated. The solubilizing ability of EDTA may vary among different excitable membranes and perhaps depends more upon the membrane than the enzyme; particulate enzyme in sarcolemma appeared to be unaffected by 2.5 mmol/l EDTA while the particulate enzyme in sarcoplasmic reticulum showed increased solubilization by this treatment (Liu & Mittag, 1974).

B. Assay Methods

The three current methods used to assay AChE are (i) the pH-stat

method; (ii) the Ellman procedure (Ellman et al, 1961) and other spectrophotometric methods; and (iii) the radiometric assays.

(i) The pH-stat method This assay is an automated continuous electrometric titration of H^+ ions released during ester hydrolysis (Wilson & Cabib, 1954) and is based on earlier manual techniques (Glick, 1937; Alles & Hawes, 1940). Measurements of enzyme activity at low substrate concentrations utilizing a dual-syringe pH-stat assay has been described by Jensen-Holm (1961) and Krupka (1966).

(ii) The Ellman procedure This method is limited to thio-esters; it is a spectrophotometric method based on the reaction of the hydrolysis-product mercaptide with a chromophoric oxidising agent. In systems free of contaminating esterases, relatively nonspecific chromophoric substrates and spectrophotometric or fluorescence assays may be used (Kramer & Gamson, 1958; Prince, 1966a,b; Guilbault et al, 1968; Rosenberry & Bernhard, 1972).

(iii) Radiometric assays Several of these assays have been described which require a cationic ester of an isotopically labelled neutral acid, usually acetylcholine. The labelled hydrolysis product acid is separated from unreacted labelled ester either by ion exchange (Reed et al, 1966; Hoskin et al, 1969; Blume et al, 1970) or by phase separation (Potter, 1967; Hall, 1973) techniques.

The three methods described provide a variable which is proportional to the moles of ester hydrolyzed over any period of

examination. The pH-stat method is directly applicable to any ester which yields a proton on hydrolysis and may be used with either soluble enzyme or tissue homogenates; however, it is the least sensitive of the three methods. The spectrophotometric methods are perhaps the easiest to apply but cannot be applied to homogenates without clarification of the assay mixture, and interfering chromophores must be excluded. In addition, the spectrophotometric method requires insignificant contamination by free thiol groups other than the hydrolysis product. The radiometric assay is the most sensitive of these methods and can be directly applied to homogenates or intact tissue, however, it is not as precise as the other methods and appropriate controls are required in the presence of agents which might affect the separation or distribution of ester and acid.

C. Purification

(i) Older methods Prior to the introduction of affinity chromatography conventional methods were successful in obtaining essentially homogeneous enzyme only from the eel electric organ (Rothenberg & Nachmansohn, 1947; Lawler, 1963; Kremzner & Wilson, 1963; Leuzinger & Baker, 1967). Some of these methods were applied to the AChE from erythrocyte membranes and mammalian brain but they yielded a product with a specific activity of only a few per cent of that obtained from affinity chromatography (Berman & Young, 1971; Chan et al, 1972a). Two methods applied to the eel enzyme, extracted after partial autolysis in toluene were (1) homogenization in 5% $(NH_4)_2SO_4$ followed by three consecutive $(NH_4)_2SO_4$ fractional precipitations; these methods were

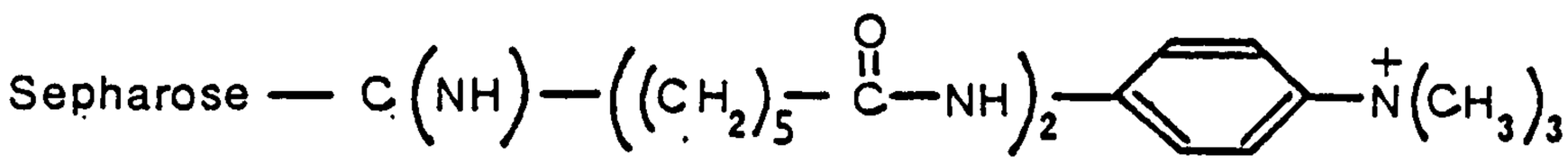
introduced by Rothenberg and Nachmansohn (1947) and modified by Lawler (1959). Recovery of the enzyme activity was about 15% and the specific activity was 20% of that later demonstrated to be maximal for essentially homogeneous 11 S AChE; (2) homogenization in 5% $(NH_4)_2SO_4$ followed by a series of chromatographic procedures introduced by Kremzner and Wilson (1963). In this case, the recovery of the enzyme activity was about 10% with a specific activity nearly equivalent to the maximal obtained for enzyme purified by affinity chromatography (Leuzinger & Baker, 1967; Rosenberry et al, 1972). Since the introduction of rapid, efficient and convenient affinity chromatography procedures, the older methods of purification appear to be rather inefficient and a detailed examination of these methods is unnecessary.

(ii) Affinity chromatography This technique of purification was introduced by Cuatrecasas et al (1968, 1971a, 1971b) and it has been of great importance in the purification of a variety of proteins. The essential considerations which arise in the application of this technique include the choice of a solid matrix, the methods of affinity ligand coupling, the characteristics of the spacer-arm, the choice of ligand, and the concentration of attached ligand.

The concept of the use of resin-bound catalytic-site ligands in the chromatography of AChE was introduced by Kremzner and Wilson (1963) and predated the practical application of the general affinity chromatography technique by Cuatrecasas and his colleagues. The benzylated-DEAE cellulose applied in their purification procedure was synthesized with the intention of preparing cellulose with an attached quaternary ammonium ligand. However, few

quaternary groups were formed (Froede & Wilson, 1971) and the resin behaved like an ion-exchange resin. Kalderon et al (1970) and Berman and Young (1971) were the first to attach affinity ligands to either 2% or 4% agarose resins, based on the technique of Cuatrecasas et al. These studies showed that the purification obtained were much higher than any previous one-step purification procedure when the method was applied to the purification of AChE from both eel electric organ and erythrocyte membranes.

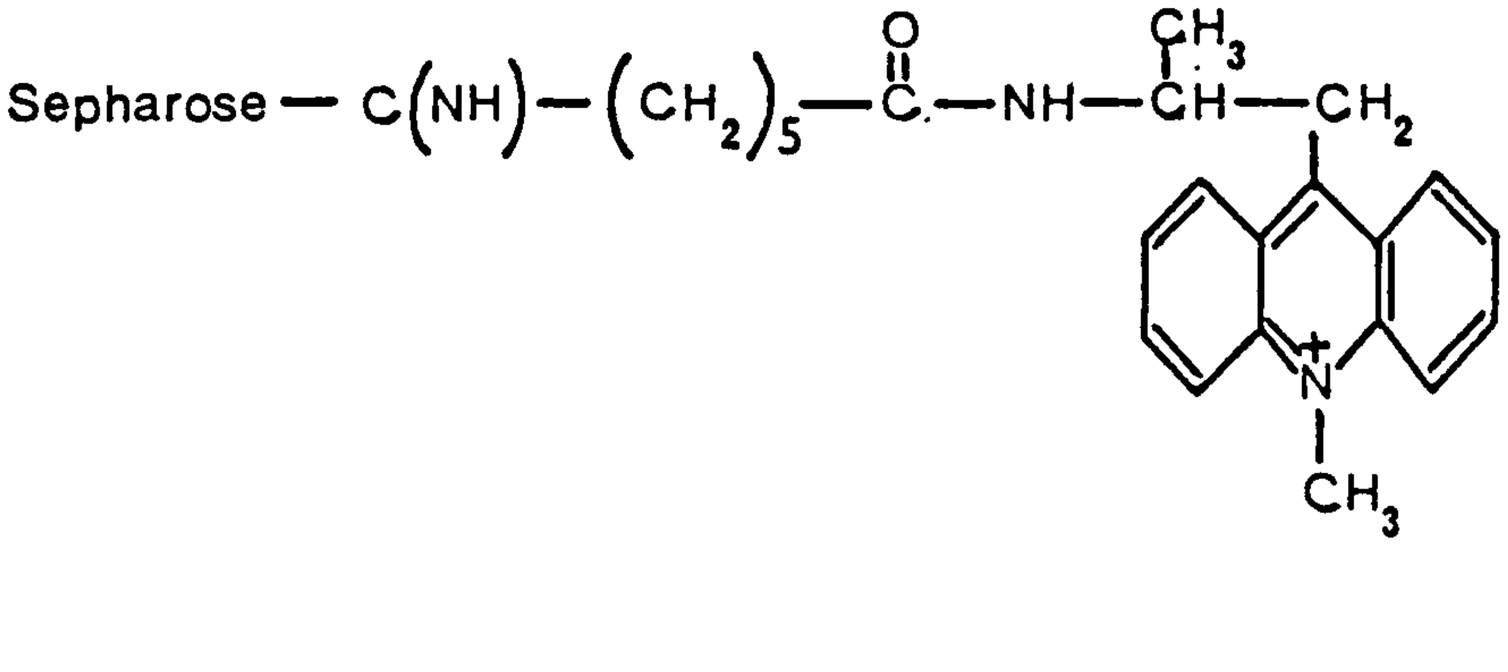
Rosenberry et al (1972) and Dudai et al (1972a) applied the affinity chromatography procedure to the purification of eel 11 S enzyme. They used the affinity ligand, N-acyl-p-aminophenyltrimethylammonium; the spacer-arm was N-(6-aminocaproyl-6'-aminocaproyl) and the resulting resin was di-(-6-aminocaproyl)-PTA-Sepharose. (shown below)



Using this affinity resin, the purification of extracts of eel 11 S enzyme from several laboratories (Dudai et al, 1972a; Berman, 1973; Chan et al, 1973) were reported to have yields ranging from 46-58% and specific activities of the purified enzyme ranging from 7.2-10.5 (mmoles ACh hydrolyzed per min. per mg. protein). In these laboratories, elution procedures were carried out using

a single concentration of an active site competitive inhibitor rather than a salt gradient to elute the enzyme from the affinity resin.

Kalderon et al (1970) used extracts which consisted primarily of the aggregative eel enzyme species, the 70-100 S species. These species were adsorbed poorly to mono (6-aminocaproyl)-PTA-Sepharose resin and appeared to be associated with other components when adsorbed (Dudai et al, 1972a). Chen et al (1973) used di(6-amino-caproyl)-PTA-Sepharose at low ionic-strength in their purification experiments and they showed that the aggregative enzyme species could be purified but were not retained as tightly as the 11 S form. Consequently, Dudai et al (1972b) constructed an affinity resin with the ligand 1-methyl-9-aminoacridinium attached to an appropriate spacer-arm via the 9-amino group; this ligand maintains a high active site affinity even in the presence of 1 mol/l NaCl. This affinity resin is shown below.



When 1 mol/l NaCl extracts of fresh eel electric organ were applied to a column of this resin, followed by washing and elution with 20 mmol/l decamethonium bromide in 1 mol/l NaCl, a 50% recovery of the enzyme activity and a specific activity of the enzyme corresponding to about 60% of the maximal observed with the 11 S enzyme was obtained. Rechromatography under the same conditions increased the specific activity to about 80% of this maximal value and in addition, the purified enzyme had the same distribution of aggregative species as the crude enzyme. The results also demonstrate that the physical differences between the different species of enzyme could lead to a change in some aspects, in this case, their adsorption to affinity resins at low ionic-strength varied. These differences appeared despite the facts that aggregative and nonaggregative enzyme forms appeared to have the same K_m with acetylcholine (Massoulié & Rieger, 1969) and that no differences in inhibitor dissociation constant (K_I) values among these enzyme species had been reported. This observation emphasizes the importance of a knowledge of the distribution of enzyme species both before and after a purification procedure for an accurate assessment of the procedure and the product.

Mammalian AChE from both bovine erythrocyte membranes and bovine brain caudate nuclei has also been purified by chromatography procedures similar to those mentioned above. The crude commercial erythrocyte preparation was purified about 2500-fold with a 50% activity recovery and a specific activity about 20% that of the maximal eel 11 S value was obtained (Berman & Young, 1971; Berman, 1973). The partially purified enzyme from brain, obtained by extraction with 1 mmol/l EDTA, was purified about

700-fold with a 60% activity recovery and subsequent gel filtration on Sephadex G-200 gave an additional 3-fold purification with a specific activity about 90% that of the maximal eel 11 S value (Chan et al, 1972a).

(a) Spacer arm Cuatrecasas and Anfinsen (1968, 1971b) have emphasized the requirement that the affinity ligand be extended away from the agarose matrix for efficient interaction of the ligand with the protein active site. Steers et al (1971) have shown that interactions of the affinity ligand with the target protein on lengthening the spacer arm have increased remarkably with β-galactosidase. The retention of eel 11 S AChE on an affinity resin was observed to increase significantly when the N-succinyl-3,3'-diaminodipropylamine spacer arm was doubled (Berman & Young, 1971). However, O'Carra et al (1973) have questioned the attribution of spacer arms to a relief of steric hindrance to interaction between the ligand and the protein active site. They showed that resins with spacer arms without the attached affinity ligand strongly bound certain proteins, for example, β-galactosidase. The adsorbed β-galactosidase could not be eluted with added substrate. Therefore it appears desirable to be able to assess the active site affinity of the affinity resin independently of its retention of protein in column chromatography.

(b) Choice of affinity ligand Ideally, the interaction of the target protein with the affinity ligand at the protein active site is the only interaction between the resin and the protein. In an affinity column this interaction is reflected in the retardation factor (O'Carra et al, 1973) of the target protein,

a parameter which may be expressed in terms of the number of column volumes of buffer wash required to elute the protein in the absence of a specific eluting agent. O'Carra et al (1973) have shown that under ideal conditions of interaction, optimal retardation on the affinity column could be given approximately by the molar concentration of the attached ligand divided by K_I, the dissociation constant of the ligand interaction with the protein. In principle this is the ratio of bound to unbound protein. If specific active site competitive inhibitors are used to elute the column, retardation factors higher than 10 may be permissible. Dudai et al (1972b) used an enzyme extract with a retardation factor of about 1000, for the affinity chromatography of the aggregative AChE species on the methylacridinium resin and the elution was carried out with a competitive inhibitor at a concentration approximately 1000 times its K_I. Under these conditions, competition between the free and attached inhibitors decreases the theoretical retardation factor to between 1 and 2, and this was observed by Dudai and his colleagues. Thus such competition is a criterion for the absence of significant nonspecific interaction between the protein and the column.

Within these general requirements imposed by the retardation factor, the choice of an affinity ligand is more dependent on its specificity than on its affinity; greater specificity providing greater resolution from resin-bound contaminants and ligands which have high nonspecific affinity for proteins should be avoided.

(c) Concentration of attached ligand Ashani & Wilson (1972) have reported the existence of a noncharged phosphorylating affinity ligand although affinity ligands for AChE are usually

positively charged. If the attached concentration of charged affinity ligand exceeds a certain limit, the column will have ion-exchange properties and bind proteins nonspecifically (Kalderon et al, 1970; Rosenberry et al, 1972; Dudai et al, 1972a). Rosenberry's laboratory have set an upper limit for affinity resins to be 1.0 mmol/l attached ligand. Below this limit, they state that insignificant contaminants are bound. In their purification of AChE, when elution was carried out with a salt gradient, the concentration of attached ligand appeared to be the single most important factor. At that concentration of attached ligand the affinity resin had a reasonable capacity for the enzyme. They observed that the maximum capacity of the 11 S enzyme for resins with 0.5-1.0 mmol/l attached ligand was about 1 enzyme tetramer bound per 100-200 ligand sites, or about 1.5 mg. enzyme per ml. of packed gel.

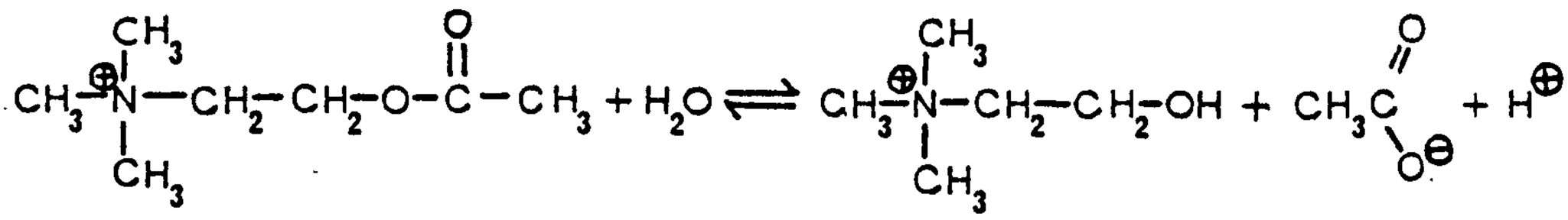
The ligand could be attached to Sepharose by two different methods (Cuatrecasas & Anfinsen, 1971b). One method is to attach spacer arms to the gel prior to condensing the affinity ligand with the spacer arm; the second method is to synthesize the spacer arm-ligand conjugate and attach it in a final step to the resin. The first procedure is synthetically easier as gel adducts are easily separated from soluble reaction by-products, and the second procedure allows more precise control of the attached ligand concentration (Rosenberry et al, 1972) and leaves no unreacted spacer arms which could bind protein nonspecifically (O'Carra et al, 1973).

(d) Evaluation of procedure There are numerous advantages for the use of affinity chromatography in the purification of AChE

and the effort spent in preparing the affinity resins is small compared to that saved by avoiding the multistep methods of older purification techniques. In addition, the affinity column can be reused several times, with very minor changes in elution profile, by regenerating the column with one to two column volumes of 6 mol/l guanidine hydrochloride prior to reequilibration in solvent buffer (Chen et al, 1974). This technique also allows large-scale purification of enzyme to proceed at the fastest pace possible and so minimize proteolytic degradation of the enzyme species. Finally, affinity chromatography appears useful not only for the quantitative purification of AChE but also for its qualitative fractionation.

4. CATALYTIC MECHANISM

Acetylcholinesterase is classified as a serine hydrolase together with other esterases and peptidases which show essentially irreversible active site phosphorylation (Cohen & Oosterbaan, 1963). Determinations of the equivalent weight and peptide analysis show that phosphorylation occurs only at a single serine residue and the amino acid sequence about this residue is very similar to the enzymes in this class (Cohen & Oosterbaan, 1963; Schaffer et al, 1973). AChE preferentially hydrolyzes esters containing a cationic group (alkylammonium group) and acetylcholine is the best substrate among these esters. The hydrolytic process is shown by the equation:



The catalytic action of AChE is due not to a coenzyme but to the structure of the enzyme protein itself (Engelhard et al, 1967). As the protein molecule folds, certain amino acid residues at distant sites of the chain are brought close to one another, forming an active centre. The active centre comprises two active sites: one fixes the substrate and is chiefly responsible for the specificity (anionic site), and the other is responsible for the cleavage

of the substrate (esteratic site).

A. The Hydrolytic Process

The hydrolytic enzyme action occurs in two stages and may be expressed in the following way:

$$E + S \rightleftharpoons ES \longrightarrow E + \text{products}$$

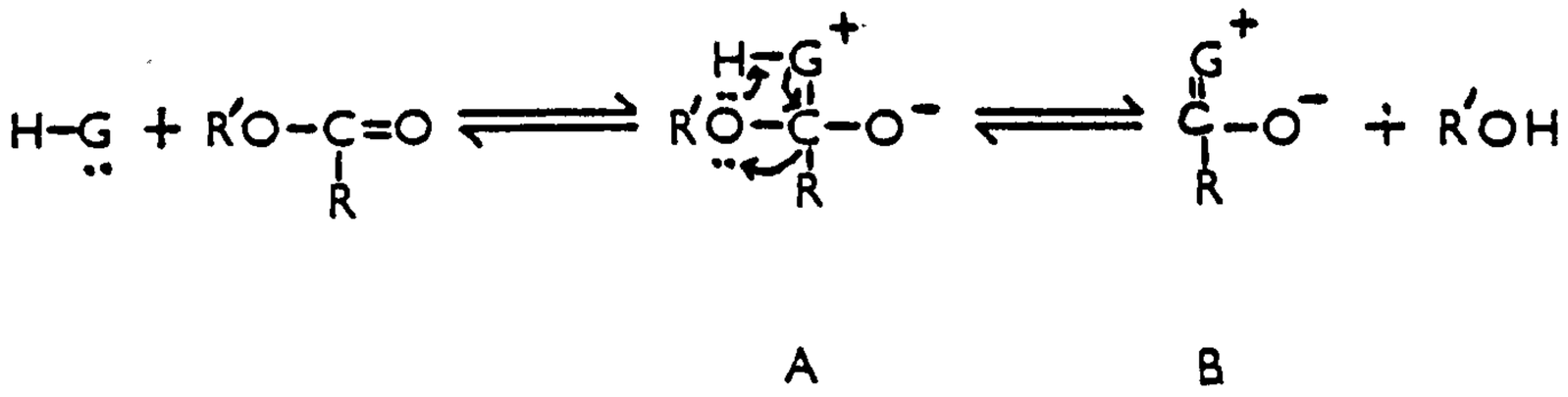
In the first phase the enzyme (E) combines with the substrate (S) to form the enzyme-substrate complex (ES), the generally postulated Michaelis-Menten complex. In the second phase the hydrolytic process takes place. The formation of an enzyme-substrate complex is today generally postulated. It is difficult to conceive how an enzyme could function without interaction with a reactant, although this interaction need not result in the formation of a stable intermediate. The assumption of a stable enzyme-substrate complex is valid for those cases where the apparent dissociation constant is small. The distinction between a critical or transition complex and an enzyme-substrate complex is one of stability; the former lies at a thermodynamic potential energy maximum and the latter at a minimum. The dissociation constant for the acetylcholine-acetylcholinesterase complex is about 1×10^{-4} and, hence, the concept of a stable reversible complex is applicable to this system (Nachmansohn, 1975).

Wilson et al (1950) proposed a mechanism for the hydrolytic

process and the mechanism is shown in Fig. I.6. The proposed mechanism assumes a process taking place in two consecutive steps. The first step is the acetylation of the enzyme with simultaneous elimination of choline. In Fig. I.6, (A) shows the Michaelis-Menten complex; (B) shows the acylated enzyme depicted as enolate ion (one of the resonance forms); and (C) is an acid-enzyme complex similar to the ester-enzyme complex and leads to regenerated enzyme and acetic acid. The mechanism follows from the enzyme-substrate complex structure and assigns a positive role to the enzyme in effecting a combined acid-base attack. The acetylenzyme reacts with water or other nucleophilic agents, such as hydroxylamine, or an alcohol, eg. choline, to give an acid or an ester. It is possible to start with acids or esters, but only the undissociated acid molecules have the electrophilic carbon atom necessary for the enzyme-substrate complex. At pH 7 the fraction of undissociated acid molecules is small and so the intermediate is far more rapidly formed from esters than from carboxylic acids. Wilson et al (1950) compared the enzyme-catalyzed formation of hydroxamic acid and choline esters from simple esters and the corresponding acids, and the reaction with the esters was found to be about 100 times faster.

The mechanism proposed has been confirmed in many ways (Wilson, 1951a,b). Wilson found that H_2S is evolved and acetic acid is formed, as predicted by theory, when thioacetic acid was used as substrate. The reaction is completely inhibited by prostigmine. In his studies on the reaction of thioacetic acid with the enzyme, Wilson was also able to show that the anionic and esteratic sites are both functionally and spatially separated.

Fig. I.6 The Mechanism of the Hydrolytic Process
(Wilson, Bergmann and Nachmansohn, 1950)



When a small-sized cationic inhibitor, such as trimethylammonium ion, is used, the hydrolysis of ethyl acetate is completely inhibited. However, the hydrolysis of thioacetate, even at high inhibitor concentrations, is inhibited up to a maximum value of 30%. From these observations, the binding of the small molecule to the anionic site apparently does not interfere markedly with the reaction at the esteratic site, provided the reacting molecule is as small as thioacetate.

The evidence of oxygen exchange between acids and water, as postulated by theory, shown with the use of isotopic oxygen (O^{18}), has shown further support for the mechanism (Sprinson & Rittenberg, 1951; Bentley & Rittenberg, 1954). Stein and Koshland (1953) have also confirmed the theory following their own observations.

Wilson's idea of an acylated enzyme being the intermediary form in the hydrolytic process has also been widely accepted by many enzyme chemists in the analysis of various enzymatic mechanisms.

Interpretation of kinetic studies in terms of mechanistic processes has brought about many modifications to the original model proposed by Wilson et al (1950). The most important contributions have been provided by Brestkin and Rozengart, 1965 (see Fig. I.7); Krupka and Laidler, 1961b; Krupka, 1964, 1966a,b, 1967 (see Fig. I.8).

Brestkin and Rozengart (1965) proposed that only one histidine is involved in the reaction mechanism, whereas Krupka (1967) proposed that two imidazoles are necessary to account for the two pK values (5.6 and 6.3) which he observed in his studies. In both mechanisms, an integral part is concerned with a rearrangement in the active site and a major difference is in the mechanism of

acyl-enzyme formation. Brestkin and Rozengart (1965) explained that the Michaelis-Menten complex (2b, Fig. I.6) led directly to covalent bonding between serine oxygen and carbonyl carbon (3, Fig. I.7). However, Krupka (1966b, 1967) postulated that reaction with the substrate is initially at imidazole B_2 (Fig. I.8), followed by transfer to a second group B_1, to form an acetylenzyme. Deacetylation occurs by the reverse process.

The catalytic mechanism may thus be explained as follows: (Froede & Wilson, 1971).

$$E + S \rightleftharpoons E.S \rightleftharpoons E'.P_1 \rightleftharpoons E' + P_1 \overset{+H_2O}{\rightleftharpoons} E.P_2 \rightleftharpoons E + P_2$$

where E is the enzyme, S is the substrate, E.S is the Michaelis complex between enzyme and substrate, $E'.P_1$ is the Michaelis complex between acetyl enzyme and choline, E' is the acetyl enzyme, $E.P_2$ is the Michaelis complex between acetic acid and enzyme, P_1 is choline, and P_2 is acetic acid. When P_1 and P_2 concentrations are low and insufficient to affect the kinetics, dissociation of the complexes is rapid as shown below:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.S \xrightarrow{k_2} E' + P_1 \xrightarrow[+H_2O]{k_3} E + P_2$$

Fig. I.7 Acetylcholinesterase Catalysis
(Brestkin and Rozengart, 1965)

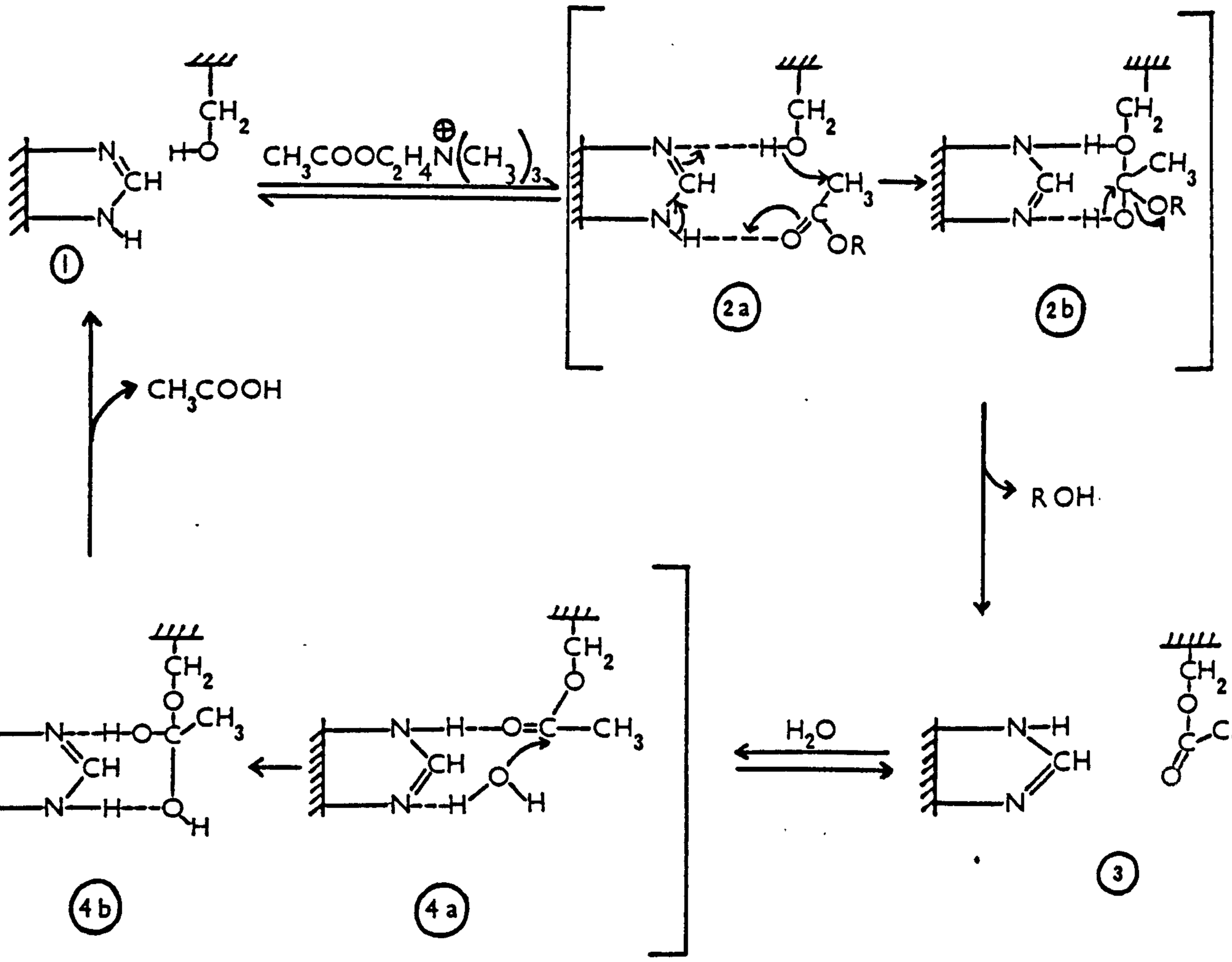
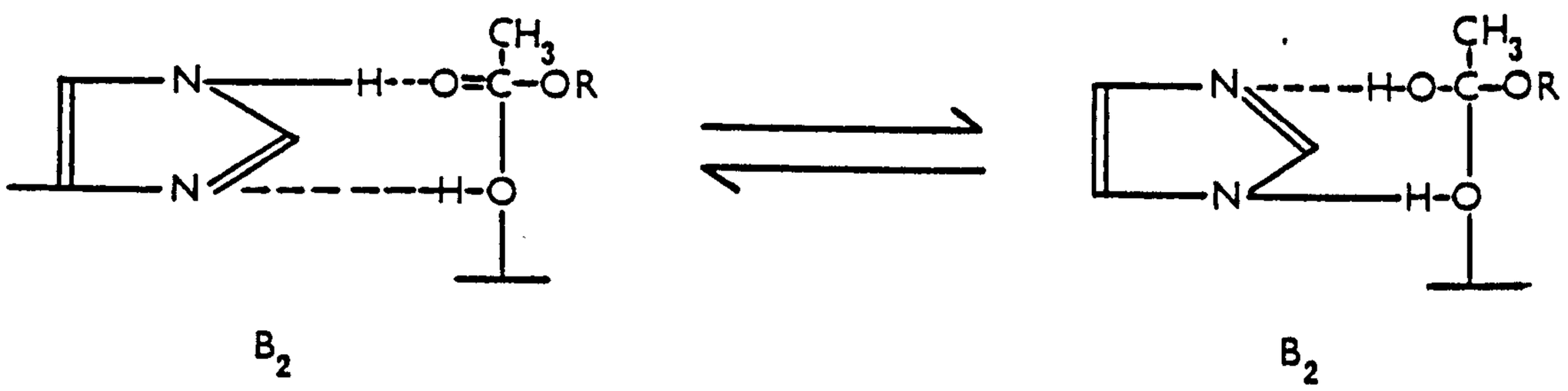


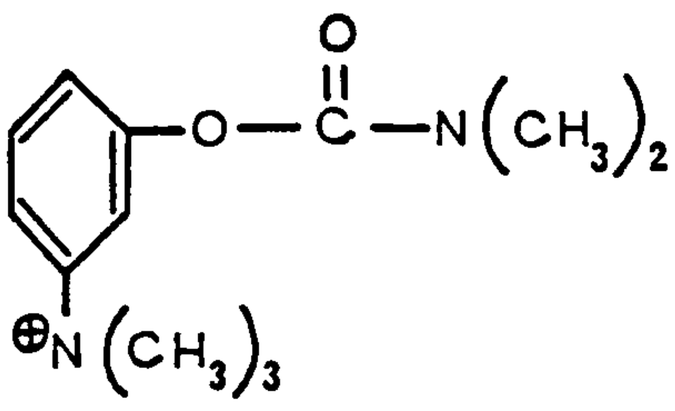
Fig. I.8 Acetylcholinesterase Catalysis
(Krupka, 1967)



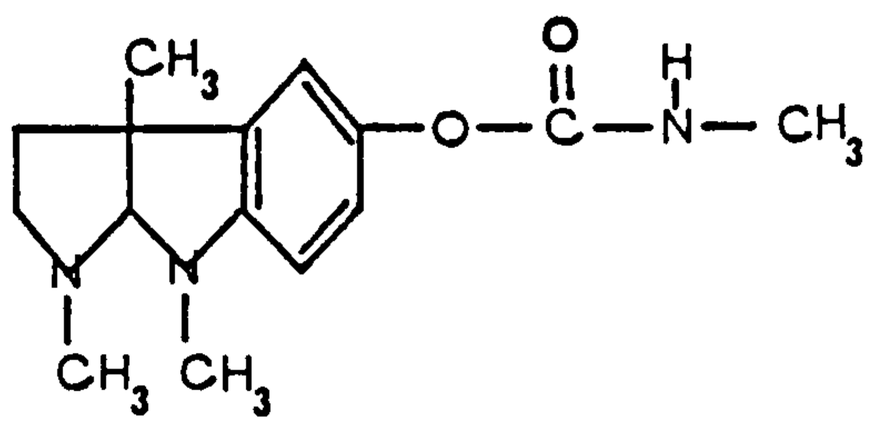
B. The Anionic Site

As mentioned before, acetylcholinesterase cleaves preferentially esters containing a cationic group (alkylammonium group). This preference for substrates containing a cationic group suggests the presence of an anionic site in the active centre (Wilson & Bergmann, 1950a; Adams & Whittaker, 1950; Myers, 1950; Wilson, 1952).

Further evidence for an anionic site has emerged from experiments with reversibly cleavable inhibitors. Wilson and Bergmann (1950a) demonstrated the presence of an anionic site in AChE with the competitive inhibitors physostigmine (eserine) and prostigmine. Prostigmine is a quaternary ammonium ion and consequently is positively charged at all pH's, whereas, physostigmine is a tertiary amine and changes from conjugate acid at pH 6 to a neutral molecule at pH 10. Therefore, the inhibitory action of physostigmine is reduced when the cationic group is converted into a neutral one while prostigmine does not lose its inhibitory action under the same conditions.



Prostigmine



Physostigmine

The effect of inorganic ions on substrate hydrolysis and inhibition also support the existence of an anionic site. Wilson (1960) demonstrated that the inhibition by quaternary ammonium ions is decreased drastically by increased ionic strength and that acetylcholine hydrolysis is also increased by the presence of salts.

Other forces participating in the fixation of the substrate besides Coulombic ones are van der Waals forces involving the methyl groups of choline and uncharged substrates (Wilson, 1952; Bernhard, 1955; Friess et al, 1962). Wilson (1952) observed the effect of binding at the anionic site when he used the competitive inhibitor, hydroxylthylammonium at pH 7 (cationic). He reported that each alkyl group increased binding seven-fold except for the fourth methylation and so he assumed that this increased binding was due to van der Waal's attraction by the methyl groups to hydrocarbon moieties in the enzyme (hydrophobic bonding).

Degradation studies have shown that ω-carboxyl groups of aminodicarboxylic acids are the most likely carriers of negative charge on the anionic sites, and such studies have shown that the active centre in AChE involves glutamic acid (Sanger, 1963; Oosterbaan & Cohen, 1964).

C. The Esteratic Site

According to Wallenfels and Streffer (1964), enzymatic catalysis often relies on acid-base catalysis. This necessitates the presence, in the esteratic site, of an 'acidic' and a 'basic' active group, whose function in the enzymatic reaction depends on the degree of protonation and thus on the pH of the medium. Wilson and Bergmann (1950a,b) have inferred the existence of such active

groups in AChE, from bell-shaped activity versus pH curves, which show a diminished activity above and below the optimum pH.

The decrease in activity in acidic medium is due to inactivation of the basic active group by protonation. Investigations of the pH-dependence of the maximum cleavage rate of charged and uncharged substrates by Krupka (1966) showed that the basic group is composed of a first (B_1) and a second (B_2) group (pK 6.3 and 5.5, respectively). The activity drop in alkaline medium is attributable to the inactivating deprotonation of the acidic group AH (pK 9.2) (Wilson, 1951a; Laidler, 1955; Krupka & Laidler, 1960).

Several findings indicate that serine, which contains an OH group and is present in all hydrolases, plays an important role in the esteratic site (Koshland, 1964). Evidence for the existence of a serine residue in the esteratic site has been obtained by labelling with $DF^{32}P$ and subsequently degrading the enzyme, yielding O-diisopropoxyphosphoryl serine (Schaffer et al, 1954; Aldridge, 1957). The amino acid sequence was elucidated for tripeptide units in acetylcholinesterase (Sanger, 1963) and it is -Glu-Ser-Ala- while the amino acid sequence for the active site of cholinesterase is -Phe-Gly-Glu-Ser-Ala-Gly-(ala,ala,ser) (Oosterbaan, 1967).

Under physiological conditions, free serine does not catalyze the hydrolysis of esters (Rozengart, 1961), nor does it react with alkyl phosphates (Wagner-Jauregg & Hackley, 1953; Ashbolt & Rydon, 1957). Therefore, the serine must be activated by other amino acid groups in the enzymes. The imidazole group of histidine probably represents such an activating group (Bergmann et al, 1956) and this group is known to possess several biochemical and catalytic

functions (Barnard & Stein, 1958). By analogy with chymotrypsin and trypsin, one may assume that each of the two basic groups B_1 and B_2 consists of an imidazole residue and that they are brought close to each other in the protein by a conformational change (Krupka, 1966b). Various mechanisms have been proposed for the cooperative action of the active groups and according to the most probable mechanism, the doubly bonded nitrogen of the imidazole ring abstracts a proton from the OH group of serine, presumably in a concerted mechanism. The resulting serine alkoxide anion then attacks the substrate or the alkyl phosphate nucleophilically and the acetylated serine thus formed is brought into the vicinity of the second imidazole group by a configurational change. This imidazole group abstracts a proton from water and thus causes hydrolysis, ie. deacetylation of the serine. Krupka (1966b) has postulated a simple scheme for this mechanism and this is shown in Fig. I.9.

D. Peripheral Anionic Sites and the Induced-Fit Model

The existence of peripheral anionic sites on AChE would certainly be anticipated from the amino acid composition and the isoelectric pH of 5 (Chen et al, 1974). Changeux (1966) was the first to suggest that such peripheral sites may regulate activity at the catalytic site. Multi-quaternary ligands were observed to have antagonistic effects on the enzyme activity at low ionic strength; in particular, the pachycurare flaxedil (gallamine triethiodide) was observed to reverse the enzyme inhibition due to decamethonium, a leptocurare. Other members of the two curare classes generally maintained this antagonism, but flaxedil was by

Fig. I.9 Schematic Representation of the Active Site in Acetylcholinesterase (Krupka, 1966)

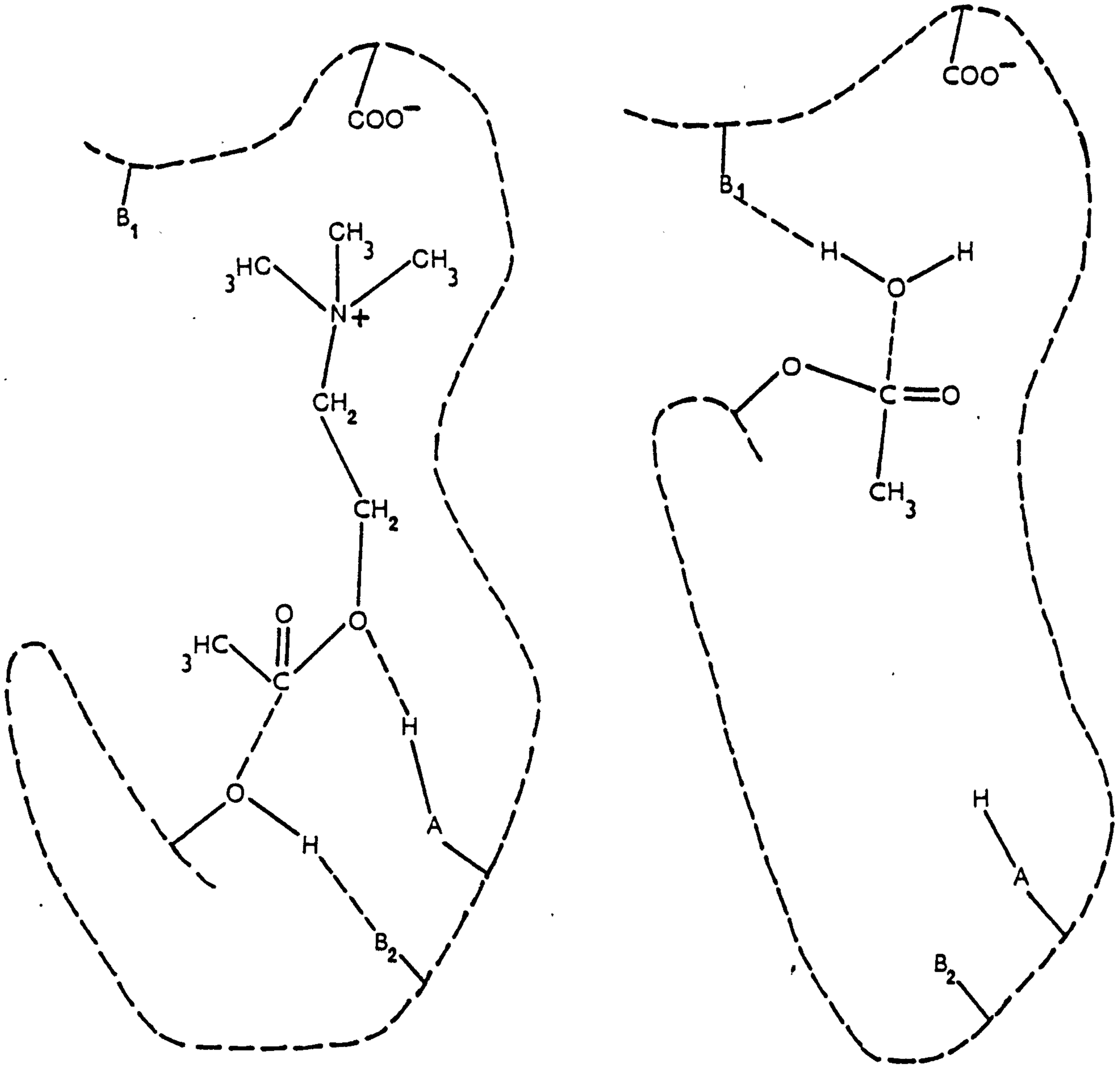


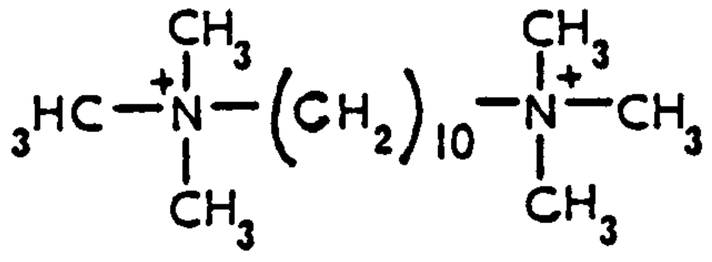
Fig. I.9 Schematic Representation of the Active Site in Acetylcholinesterase (Krupka, 1966)

B_1 and B_2 are the basic groups (pK 6.3 and 5.5, respectively), AH is the acidic group (pK 9.2), OH and COO^- are the serine hydroxide group and the anionic active site.

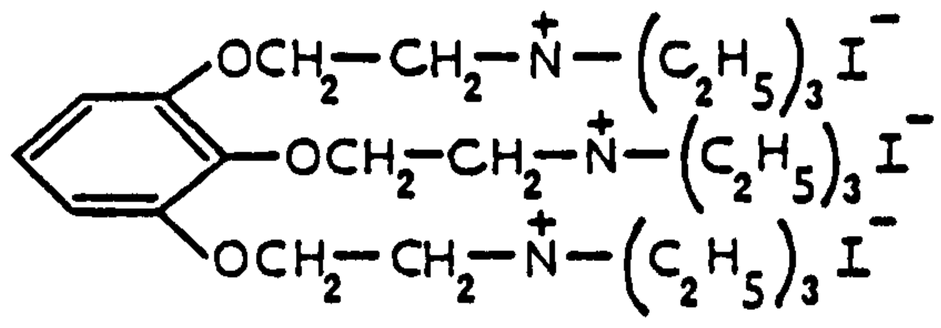
(a) Enzyme-substrate (ES) complex in the case of acetylcholinesterase

(b) Acetylated enzyme (EAc)

far the best pachycurare antagonist.



Decamethonium

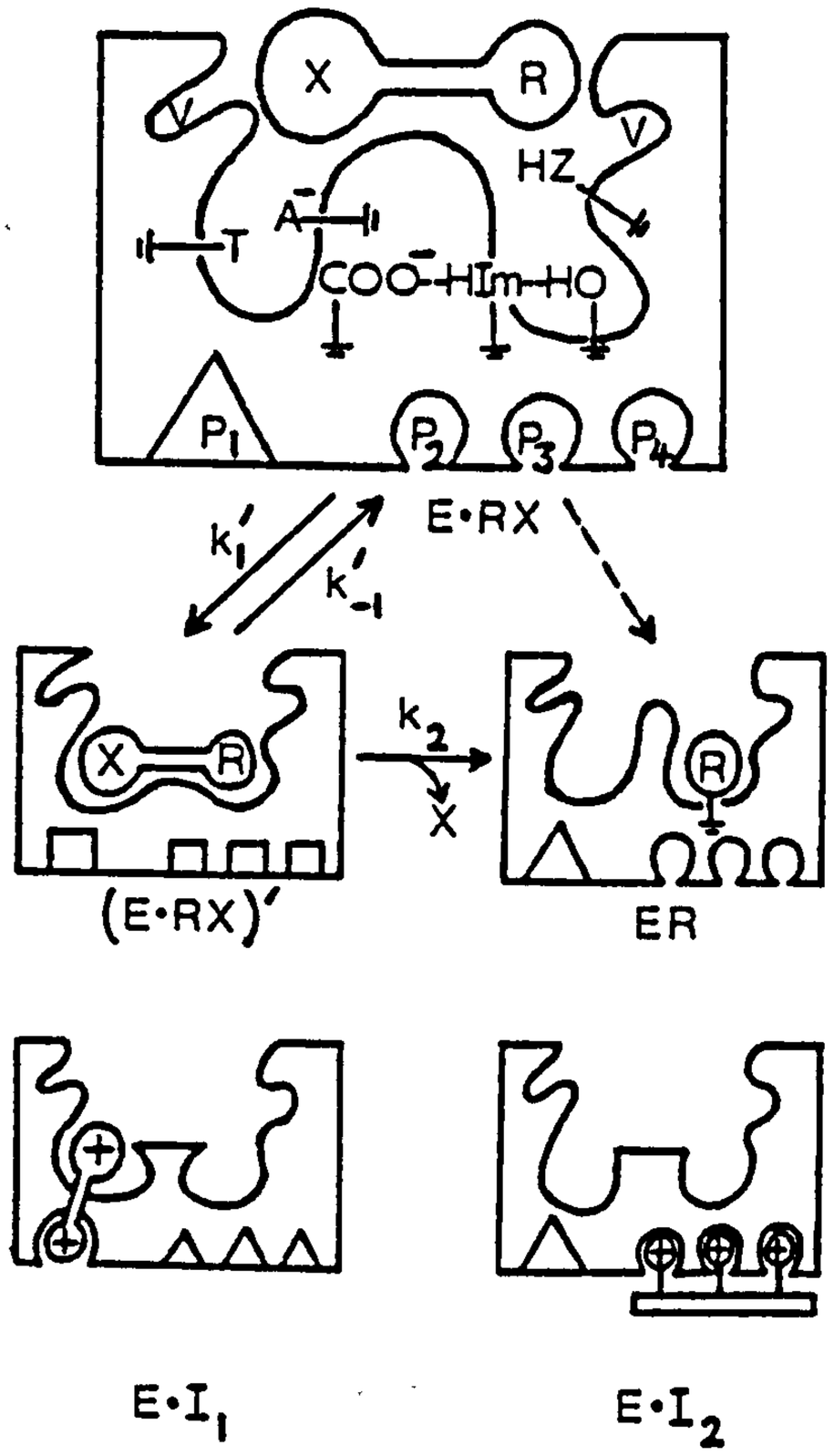


Flaxedil

Several kinetic studies were carried out and the conclusions of a few representative studies suggested that under certain conditions, catalytic activity could be modified by ligand binding at sites other than the catalytic site. The enzyme therefore meets this minimal criterion for allosteric control (Monod et al, 1965; Changeux, 1966; Belleau et al, 1970; Kitz et al, 1970; Roufogalis & Quist, 1972). This conclusion was applicable for aggregative and nonaggregative soluble enzyme species from electric organ; for soluble aggregative species from mammalian erythrocyte membranes (Crone, 1973); and for particulate species from mammalian brain (Crone, 1973). Thus although certain enzyme species undergo aggregation at low ionic-strength where the effects of peripheral sites are most apparent, the physical state of the enzyme may contribute to but does not appear critical to allosteric control.

Rosenberry (1975) has formulated an 'induced-fit model' for acetylcholinesterase (Fig. I.10), based on the induced-fit model

Fig. I.10 Diagrammatic Model of the Acetylcholinesterase Catalytic Site Based on the Induced-Fit Mechanism (Rosenberry, 1975)



The initial enzyme substrate is complex E·RX; the induced-fit complex, (E·RX)'; and the acyl enzyme, ER. The enzyme-ligand complex with bisquaternary ligands like decamethonium ($E \cdot I_1$) involves the anionic site and peripheral site P_1; the complexes formed by other multiquaternary ligands like flaxedil ($E \cdot I_2$) involve ligand binding at other peripheral sites (P_2, P_3, P_4). Identified residues at or near the catalytic site include the charge-relay complex COO^---HIm--HO; an acidic group HZ; the anionic group A^- which defines the anionic site; a tryptophan residue T near the anionic site; and adjacent hydrophobic areas V.

first postulated by Koshland (1959). According to this model, the enzyme-substrate complex E.RX is assumed to be equilibrated with free E and RX and the catalytic site conformations in the free enzyme and in E.RX are assumed to be similar. The E.RX complex searches for the induced-fit complex (E.RX)' conformation which maximizes the acylation rate by lowering the transition state for the covalent bond rearrangement in k_2. Of importance is the notion that there are many potential (E.RX)' complexes for each substrate, but the transition state energy of k_1' for each such complex varies. Consequently, the primary acylation pathway involves the (E.RX)' complex for which $k_a = k_1'k_2/(k_1' + k_{-1}' + k_2)$ is maximal. The acyl enzyme ER presumably deacylates through a pathway that involves the same enzyme catalytic groups as those in acylation. A putative induced-fit complex (ER)' may form, as suggested by the small increases of the hydrolysis of acyl enzyme which are observed with peripheral site ligands. The apparently unperturbed pH dependence of deacylation indicates that any (ER)' species must be equilibrated with ER. As suggested by the enzyme specificity, the relatively small size of acyl groups which may be accommodated at the esteratic site may be a major factor in the steric definition of the induced-fit complex. Deacylation is much slower for larger acyl groups (Froede & Wilson, 1971), suggesting that an equilibrium ratio (ER)'/ER is decreased when R is large. The acyl group conformation is also important in determining the relative deacylation rate (Bieth et al, 1973). An induced-fit pathway may be important for cationic nucleophiles which act as reactivating agents, since inorganic cations increase the reactivation rates achieved with neutral nucleophiles (Green &

Smith, 1958). Reversible ligands which bind to peripheral sites are proposed to modulate activity be reducing conformational flexibility and thus to stabilize a catalytic site conformation which lowers the transition state for acylation by poor substrates but raises it for good substrates. Such effects would be more pronounced for substrates limited by k_1' rather than by k_2. This is shown schematically in Fig. I.10 for enzyme complexes involving decamethonium ($E.I_1$) and flaxedil ($E.I_2$).

E. Steric Configuration of the Active Centre

The protein must be in a suitable steric configuration in order to permit the joint attack of the active groups, which, like serine and histidine in the amino acid chain, are not adjacent. Koshland (1963) and Neurath (1964) have demonstrated that the serine and histidine in hydrolases such as α-chymotrypsin, ribonuclease, and carboxypeptidase A, are brought together by a folding of the protein molecule fixed by disulphide bridges. According to the theory of induced fit (Koshland, 1958, 1960, 1963), the substrate also brings about a catalytically active configuration of the active centre of the protein; the ability of the protein to fit itself to the substrate may affect the substrate specificity.

The steric configuration of the active site in AChE has been obtained from experiments with substrates and reversible cleavable inhibitors. In such experiments the inhibition or the rate of cleavage was determined in order to establish which are the ones that fit best (Friess & Baldridge, 1956; Friess, 1957; Wilson & Quan, 1958; Krupka & Laidler, 1961a,b; Krupka, 1964). The protonated basic group B_1 can impede the access of a reversible inhibitor

to the adjacent anionic site. The lowering of the inhibition constant for tetramethylammonium chloride, accompanying a change in the pH in the region of the dissociation constant of the first basic group B_1, indicates that the distance from the anionic site cannot exceed 5.5 Å. Since such an effect has not been found with the second basic group B_2 and the acidic group AH, these groups do not interact with the anionic site, and are situated from it at distances of 8-10 Å and 9 Å, respectively (Krupka, 1966a,b).

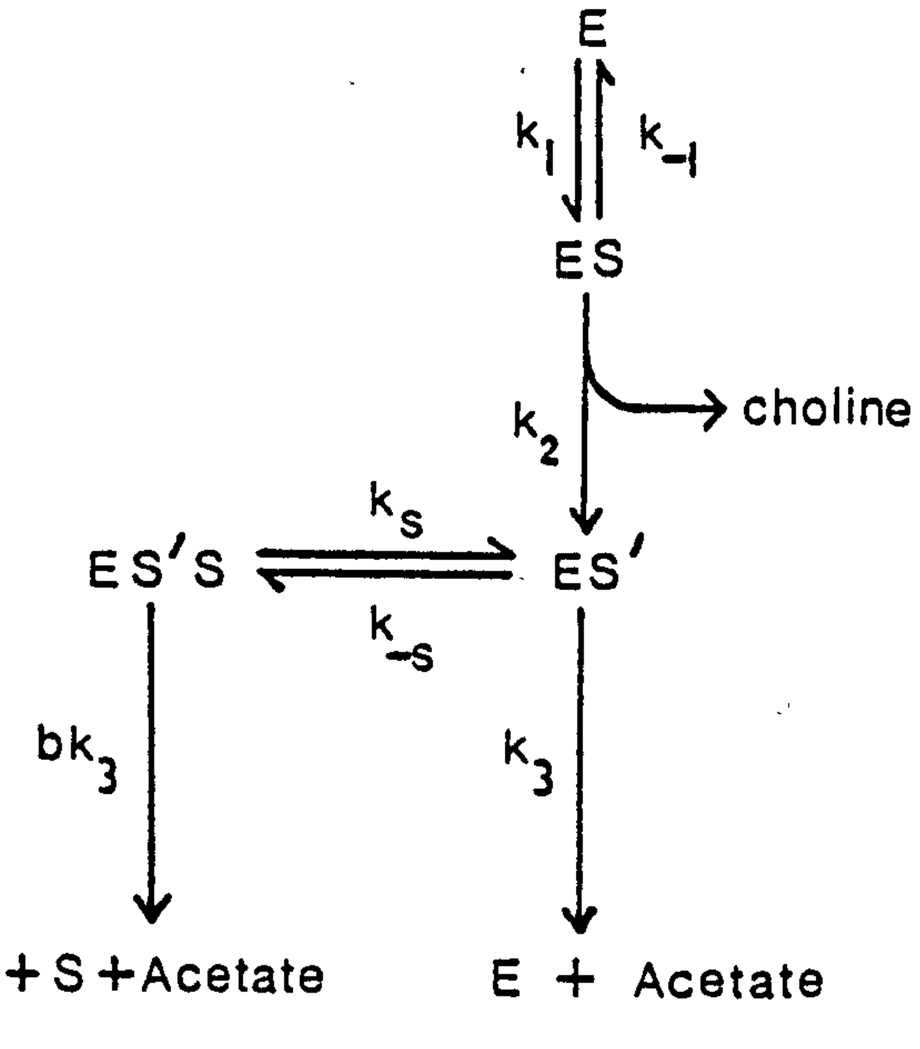
F. Inhibition Mechanisms

When the rate of hydrolysis of acetylcholine by acetylcholinesterase is plotted against the substrate concentration, the resulting bell-shaped curve shows a marked decrease in the enzymatic activity at substrate concentrations exceeding 2.5-3 x 10^{-3} mol/l, except with certain substrates, such as glyceryl triacetate. This action of the substrate is characteristic for hydrolysis by AChE, and this phenomenon may be used to differentiate between the latter and other hydrolases.

Alles and Hawes (1940), and Zeller and Bissegger (1943) attributed the inhibition to the formation of an inactive (or weakly active) ES_2 complex whose anionic and esteratic sites are occupied by two molecules of ACh, so that the steric configuration impedes the activation of the ester bond. However, the kinetic equations based on this assumption (Myers, 1952; Marmasse, 1963; Cohen & Oosterbaan, 1963) do not explain the experimental data satisfactorily.

Krupka and Laidler (1961b) proposed a more feasible theory in which the intermediate acetylated enzyme ES' whose anionic site is

unoccupied (as in the free enzyme), combines with excess substrate to form a much less active complex ES'S. This mechanism of inhibition is shown below.

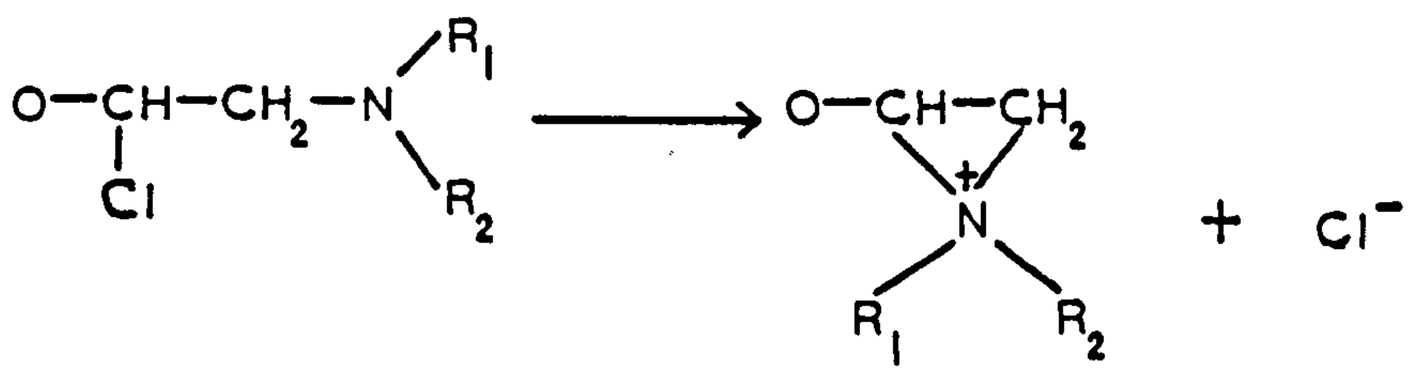


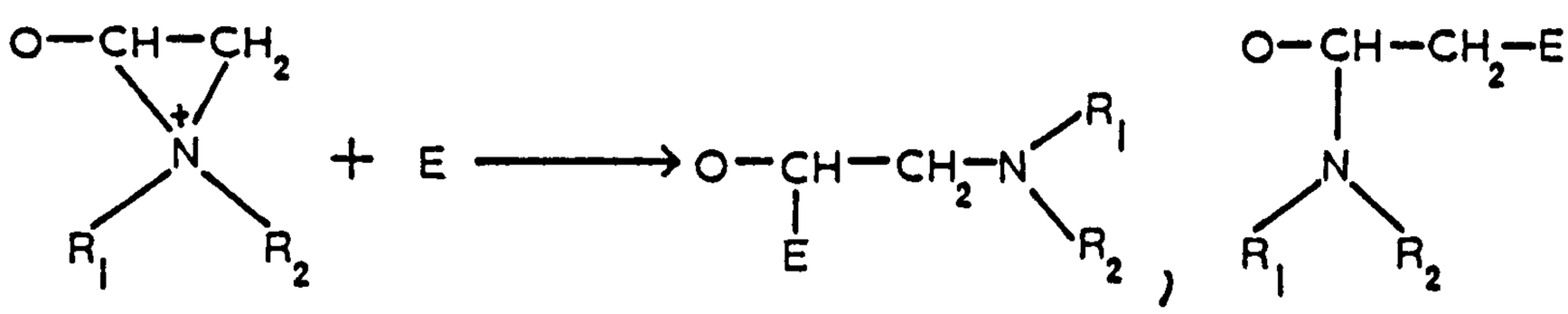
Brestkin and his colleagues (Brestkin et al, 1965) postulate that the decreased activity results from a change in the structure of the enzyme (induced-fit model) and propose that the structure of the inactive complex is $E.S_3$ or $ES'S_2$. Another possible method of inhibition could be attributed to binding at an allosteric site as proposed by Changeux (1966).

(i) Anionic site inhibitors Froede and Wilson (1971) reported that any substituted ammonium ion, especially a tertiary or quaternary ammonium ion, is a potential inhibitor since it may be capable of binding at the anionic site. In addition, the presence of long hydrocarbon chains or rings increases the binding and so is in

agreement with the postulated hydrophobic region at the anionic site.

Irreversible anionic site inhibitors have also been developed, for example, the N,-N-dimethyl-2-chloro-2 phenylamines. The active form is an aziridium ion which alkylates the enzyme at or near the anionic site (Belleau & Tani, 1966).





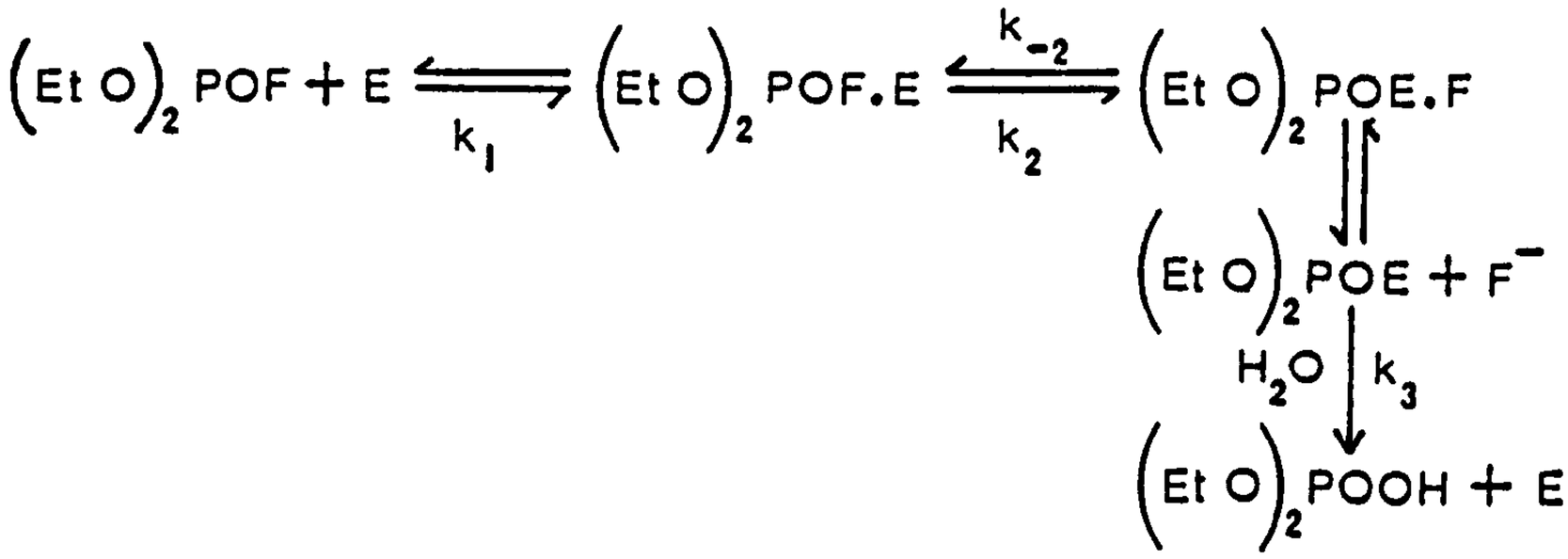
These inhibitors appear to act by preventing interaction of cationic groups with the anionic site. Wofsy and Michaeli (1967) have also reported another type of irreversible inhibitor, p-(trimethylammonium) benzenediazonium fluoroborate. This inhibitor forms a coupled azo compound.

(ii) Esteratic site inhibitors The group of compounds classified as acid transferring inhibitors (Wilson, 1971) inhibit

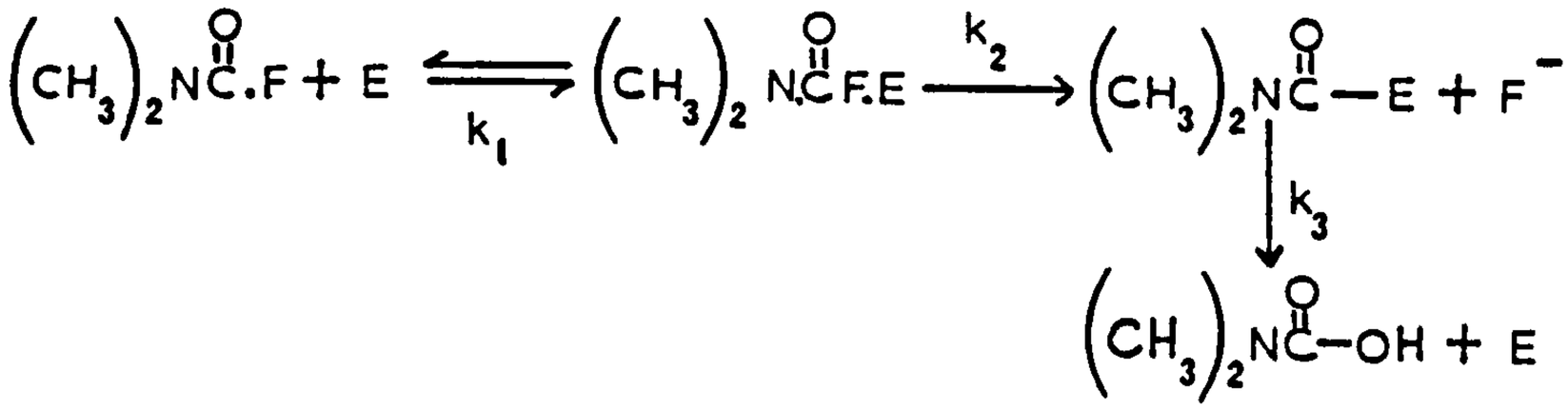
acetylcholinesterase by a reaction which occurs at the esteratic site. These compounds include organophosphates, carbamates, and sulphonates, and they react by transferring a phosphoryl group, a carbamyl group, or a sulphonyl group, to serine hydroxyl of the enzyme.

The mechanism of reaction of these inhibitors are shown below.

<u>Organophosphate</u>



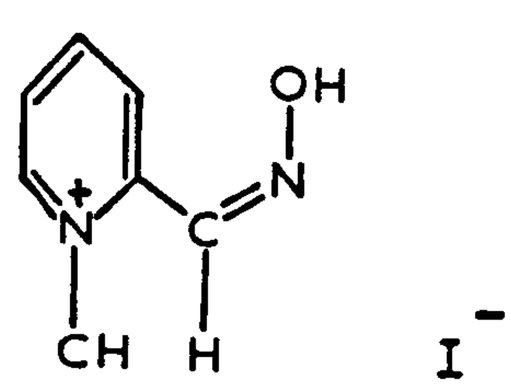
<u>Carbamate</u>



Sulphonate

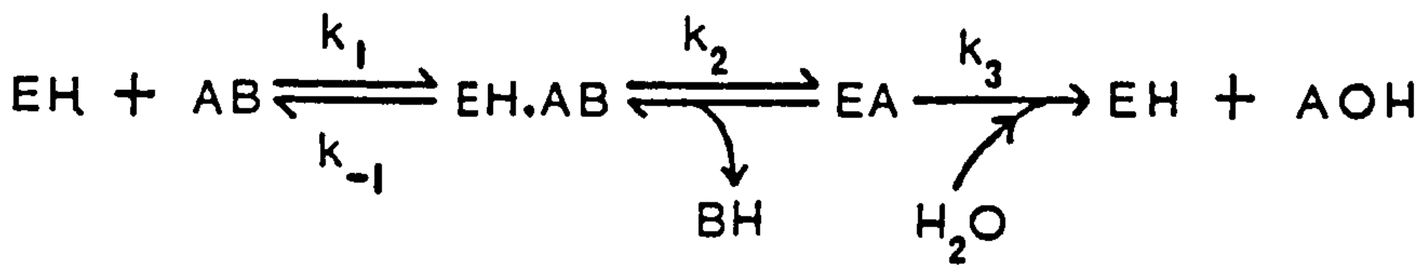
$$CH_3SO_2F + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CH_3SO_2F.E \xrightarrow{k_2} CH_3SO_2E + F^{\ominus} \xrightarrow{k_3} \approx 0$$

The reactivation of AChE by displacing the appropriate residues with nucleophilic reagents is not merely of theoretical interest, but is also the basis of an effective therapy for poisoning from these compounds. The rates of dephosphorylation, decarbamylation and desulphonylation are very slow and these rates can be increased by suitable nucleophiles. Such compounds have been developed, for example, 2-(hydroxyiminomethyl)-1-methylpyridinium iodide (2-PAM) (Wilson, 1955), which is a potent reactivator of organophosphate inhibited enzyme. The structure of 2-PAM is shown below.



(a) Mechanism of inhibition of acetylcholinesterase by organophosphorus compounds The discovery of the site of action of the organophosphorus compounds originated from secret work on their use as potential warfare agents (Saunders, 1957). In 1941, diisopropyl phosphorofluoridate was synthesized by Saunders' group and it was shown to constrict the eye pupil. By analogy with the effect of eserine, Adrian et al (1957) suggested that diisopropyl phosphorofluoridate inhibited AChE, which was then shown to be the case.

Relatively recent studies on the inhibition of AChE have been summarized by Aldridge (1971) and Wilson (1967). The essential feature of organophosphate inhibition is that they react with the enzyme in a manner precisely analogous to that of the normal substrate, the inhibitory effect resulting from the relatively long life of the phosphorylated enzyme, compared with the acetylated enzyme of the physiological reaction. The reaction of acetylcholinesterase (EH) with an inhibitor (AB), in which A is the phosphorylating group, and B is a leaving group (i.e. a group that leaves and takes no further part in the reaction), may be written:



The evidence for each step of the reaction mechanism shown above will be discussed here. It is very difficult to obtain direct evidence for the formation of a reversible complex, EH.AB, between any enzyme and either its substrate (s) or inhibitor (s), since the existence of the molecular species is transient. However, there are three lines of indirect evidence for the formation of such complexes between AChE and its organophosphorus inhibitors. (1) Reduction of inhibitory effect by the substrate - it has been realised for a long time that the presence of ACh reduces the inhibitory effect of organophosphorus compounds on AChE (Augustinsson & Nachmansohn, 1949), suggesting that competition for the same binding site occurs. (2) Structural resemblance of some inhibitors to ACh - in the organophosphorus series of compounds there is evidence that a structural resemblance of the inhibitor to the natural substrate appears to improve the inhibitory effect. (3) There is kinetic evidence that the inhibitor binds to the enzyme before forming a covalent bond with it (Main, 1969).

The formation of a phosphorylated enzyme is also supported by the following evidence. (1) Kinetic evidence indicates that after the formation of the reversible complex, EH.AB, an essentially irreversible step occurs. (2) The energy of activation of the inhibitory reaction between AChE and paraoxon has been estimated to be in the correct range for a chemical reaction, rather than a physical one such as the formation of the Michaelis complex (Aldridge, 1953c). (3) The non-phosphoryl portion of organophosphorus inhibitors are referred to as 'leaving groups' and if the reaction mechanism is correct, it must follow that BH will be

formed in an amount stoichiometrically equivalent to EA, the phosphorylated enzyme. Studies performed by O'Brien et al (1966), Bender et al (1966), Rosenberry and Bernhard (1971), and Suszkiw (1971) demonstrated that the leaving group, BH, was indeed released from the inhibitor. (4) According to the reaction mechanism, AChE samples treated with organophosphorus inhibitors having the same phosphoryl group, but with different leaving groups, should form identical phosphorylated enzymes. Several groups have demonstrated that this occurs (Aldridge & Davison, 1953; Wilson et al, 1960, 1961; Reiner & Simeon-Rudolf, 1966). (5) ^{32}P - labelled diisopropyl phosphorofluoridate forms a stable compound with AChE at the low concentrations that cause inhibition. The inhibitor is thought to combine at the active centre since the compound (phosphorylated enzyme) is scarcely formed in the presence of ACh (Michel & Krop, 1951). If the enzyme-inhibitor compound is hydrolyzed, radio-labelled phosphate and serine phosphate are recovered (Sanger, 1963), giving direct chemical proof of the formation of phosphorylated enzyme, and locating serine as the site of phosphorylation.

(b) Relation between the structure of organophosphorus compounds and their inhibitory effects on acetylcholinesterase

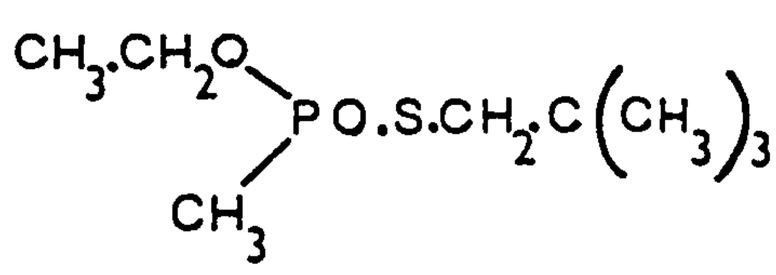
The rate of formation of phosphorylated enzyme is determined by the strength of binding between the organophosphorus inhibitor and by the subsequent rate of phosphorylation of the enzyme active site. Compounds resembling acetylcholine would be expected to have lower values for the strength of binding between the organophosphorus inhibitor, and possibly higher values for the rate of phosphorylation of the enzyme active site.

The question of whether organophosphates that inhibit AChE

really do resemble ACh structurally may be approached by regarding ACh as (i) a relatively positive hydrophobic area, joined by (ii) a spacing unit (two methylene groups and an oxygen atom) to (iii) the acetyl function, and then comparing the structure of inhibitors in relation to these three parts of the substrate.

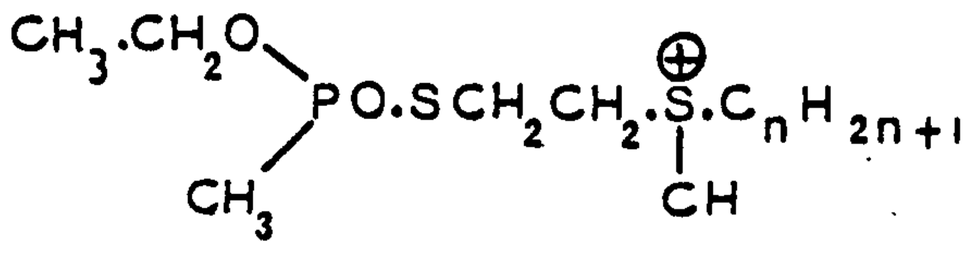
As far as activity on isolated AChE is concerned there is no doubt that a positively charged nitrogen atom increases inhibition. Metcalf (1971) quoted several examples of pairs of dimethylamino-phenylmethyl carbamates where quaternisation increases inhibitory activity on the insect enzyme by some 50-100 fold over the uncharged tertiary compound.

The N-methyl groups of ACh are attracted by hydrophobic interactions to the enzyme surface around the anionic site, and contribute more to the binding than does the positive charge on the nitrogen, at least as far as the vertebrate enzyme is concerned (Wilson, 1971). The importance of such hydrophobic interactions in organophosphorus inhibitors has been stressed by Kabachnik et al (1970). They showed that the compound:



(where the distance between the P and the tertiary C of the t-butyl group approximates to the distance between the carbonyl C and the N^+ of acetylcholine), had 90 times the overall inhibitory effect on

bovine erythrocyte AChE compared with the analogue containing merely a methyl group in place of the t-butyl. This increased activity is probably not due simply to the increased hydrophobic binding of the extra three carbon units, since the n-butyl analogue was 8-9 times less effective than the tertiary isomer shown. It is therefore likely that the isosteric nature of the t-butyl and trimethylammonium groups allows direct binding of the former to the anionic site. However, the hydrophobic patch on the enzyme near this site probably extends even further since compounds of the type:



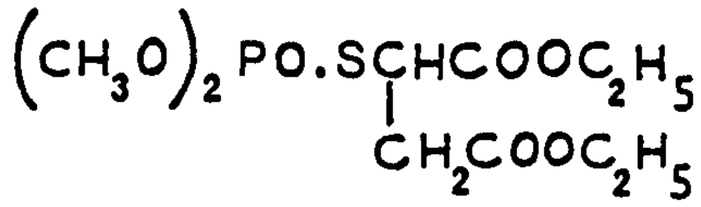
in which the positively charged sulphur atom is presumed to bind to the anionic site, show an increase in inhibitory activity as n is raised from 1 ($-CH_3$) to 6 ($-C_6H_{13}$), but not to 8 ($-C_8H_{17}$) (Kabachnik et al, 1970). Bracha and O'Brien (1968) also obtained evidence for a hydrophobic binding area near the anionic site by using carbon isosteres of amiton.

When considering the spacing portion of ACh, Chothia and Pauling (1969) estimate that the distance between the nitrogen and the carbonyl carbon atom of ACh is 0.47 nm when the substrate is in the conformation 'relevant to interaction with (bovine erythrocyte) esterase'. If organophosphorus inhibitors bind to the same places on the enzyme as the normal substrate, one would expect them to

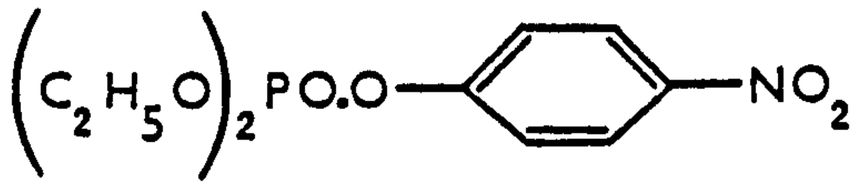
have a spacing of approximately 0.5 nm between the groups located at the anionic and esteratic centres of AChE. For the organophosphorus compound, dimethyl 3-methyl-4-nitrophenol phosphate, the distance between the phosphorus atom, which would be expected to bind to the esteratic site, and the ring methyl carbon, which would presumably bind to the anionic site, is 0.52-0.65 nm, depending on the rotation of the bonds (Hollingworth et al, 1967).

When the acetyl portion of the substrate is considered, it is observed that the organophosphorus inhibitor by definition either contains or gives rise to a P = O group which is analogous to the C = O group of ACh. It may also be noted that there is evidence (Kabachnik et al, 1970) for a hydrophobic binding area near the esteratic site.

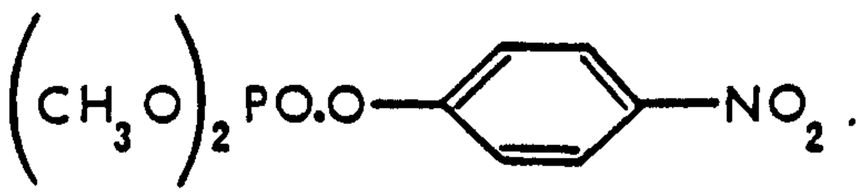
The structures of some selected organophosphorus compounds which were used in studies relevant to this thesis are shown below:



Malaoxon (and its ethyl form, ethyl malaoxon)



Paraoxon (and its isopropyl form, isopropyl paraoxon)



Methyl paraoxon

SECTION II

METHODS

1. ASSAYS

A. Acetylcholinesterase

(i) pH-stat The pH-stat is an automated system which is designed to monitor a solution for pH, to sense any change of pH and to titrate sufficient alkali to the solution to cancel out these changes. The addition of titrant is then signalled to a suitable recorder. A "Radiometer" (Copenhagen) system was used in this laboratory for the majority of kinetic measurements. There are few practical problems with this method of assay, provided the electrode assembly is carefully maintained and the pH-meter standardized prior to each set of assays. The erratic functioning observed by Jacobsen et al (1957) occurred periodically resulting in a 'stepping' effect in recorder traces and this could usually be traced to the electrode system. The assay by the pH-stat depends on the continuous and automatic titration of H^+ ions liberated by ester hydrolysis (Wilson & Cabib, 1954).

The technique is based upon an earlier manual technique whereby a constant pH was maintained by the addition of sodium hydroxide from a manually operated burette (Glick, 1937).

In the modern apparatus a glass-calomel electrode system connected to a pH-meter registers the pH of the reaction medium.

This in turn automatically directs the addition of sodium hydroxide from a burette into the reaction vessel to keep the pH at a constant predetermined value. The volume of sodium hydroxide consumed is monitored by a pen recorder. No buffer is necessary in the assay medium although with highly active membrane preparations of enzyme it is advisable to have at least a low concentration of buffer present in order to reduce the effects of a pH gradient at the membrane surface (Silman & Karlin, 1967).

AChE was routinely assayed by adding 0.3 ml. of enzyme sample to 7.4 ml. of 0.15 mol/l.NaCl + 1.3 mmol/l.$MgCl_2$ and measuring the spontaneous release of H^+ ions for 5 mins. at 30°C. and pH 7.9. After this, 0.3 ml. acetylcholine iodide was added to give a final concentration of 1 mmol/l. and the enzyme activity measured for a further 5 mins. When kinetic measurements were made at low concentrations of substrate below 5 μmol/l., a second burette containing 20 mmol/l acetylcholine iodide was employed so that a constant substrate concentration was maintained for a sufficiently long time to keep the titration curve rectilinear or only slightly curved (Jensen-Holm, 1961).

The standard conditions used for the assay of acetylcholinesterase in porcine brain and rat muscle were predetermined on preparations of homogenate and solubilized enzyme.

The pH optimum for both porcine brain preparations was in the region pH 7.9 - 8.5 (Fig. II.1). In the case of rat muscle acetylcholinesterase, the pH optimum for both preparations was in the region pH 7.6 - 8.3 (Fig. II.3). The homogenate preparations of both porcine brain and rat muscle showed a narrower pH optimum range and a reduction in high pH inhibition was observed. This may result from microenvironmental effects (Silman & Karlin, 1967) in

Fig.II.1 pH-Dependence of Acetylcholinesterase Activity in Porcine Brain

(Relative activity = Activity relative to maximum)

(a) Homogenate

(b) Sodium deoxycholate extract

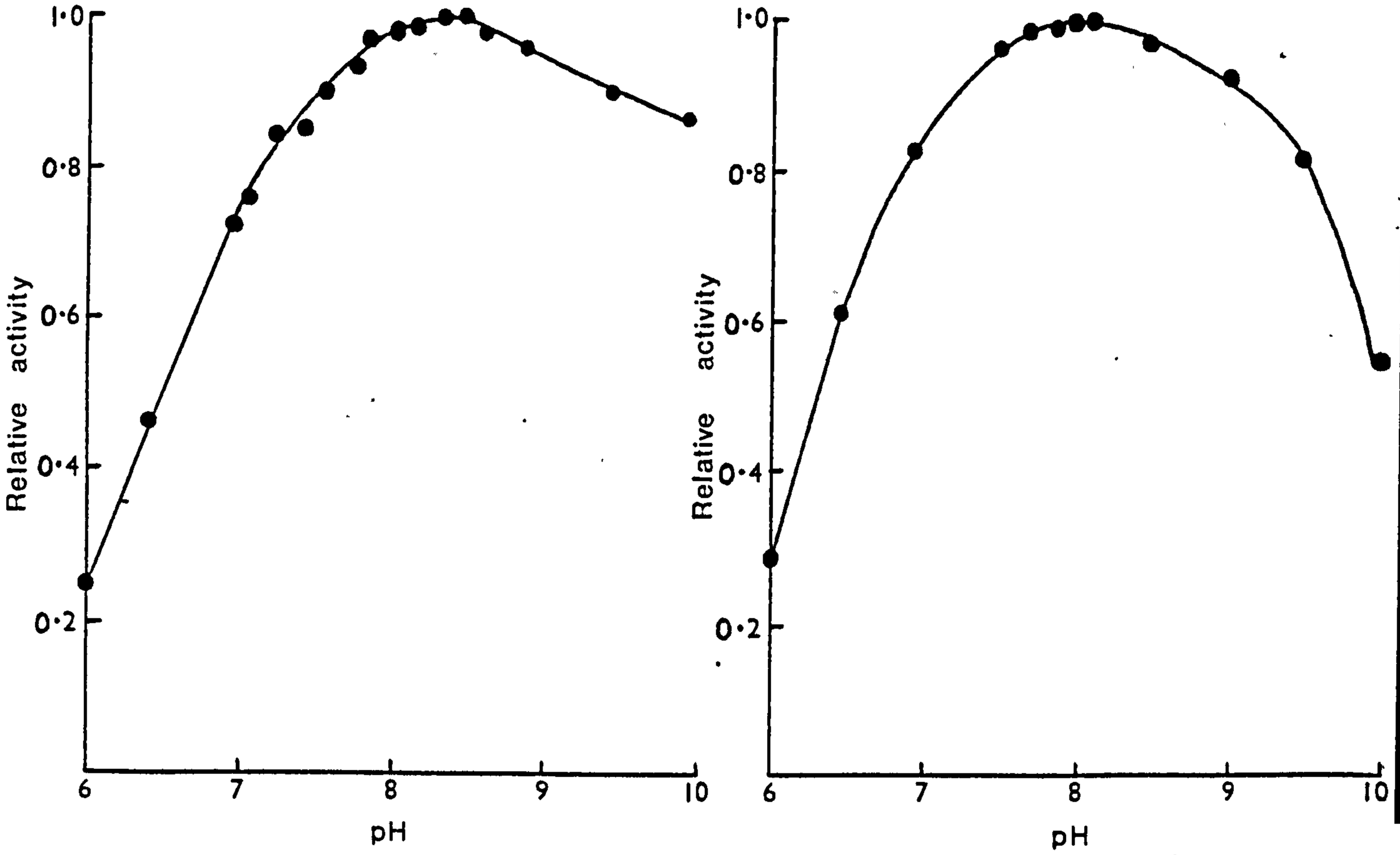


Fig. II.2 Substrate Dependence of Acetylcholinesterase in Porcine Brain

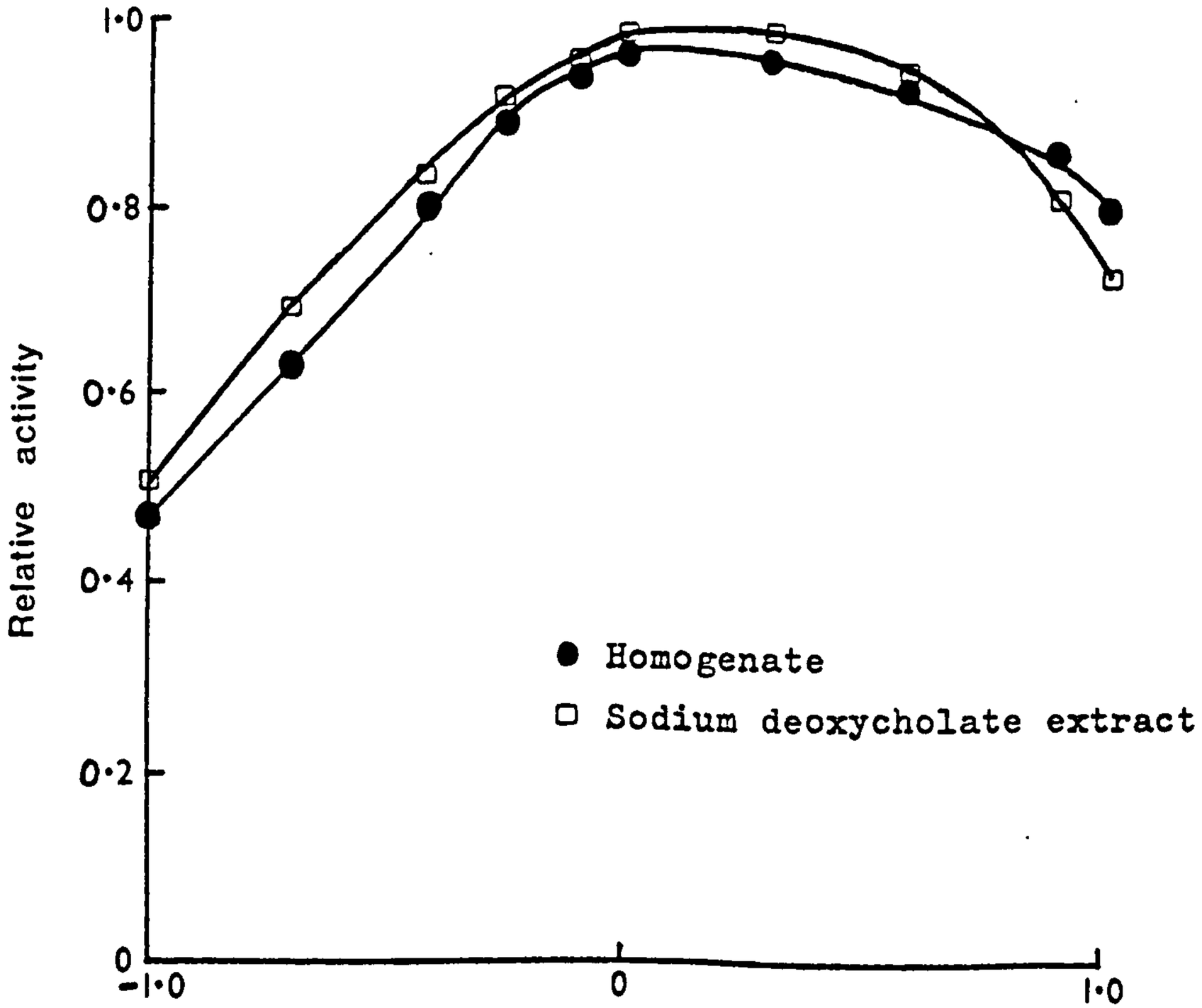


Fig. II.3 pH-Dependence of Acetylcholinesterase Activity in Rat Muscle

(Relative activity = Activity relative to maximum)

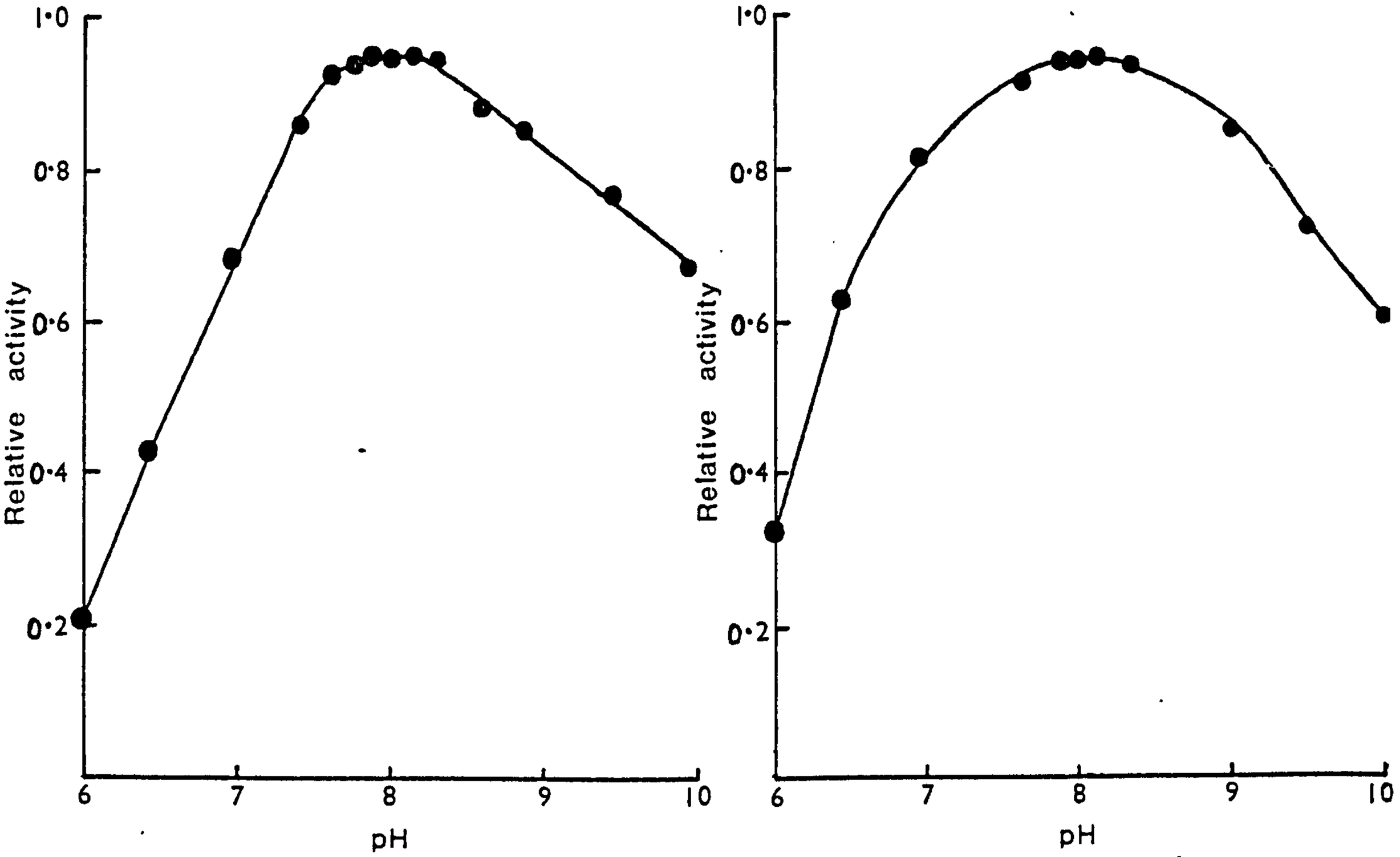
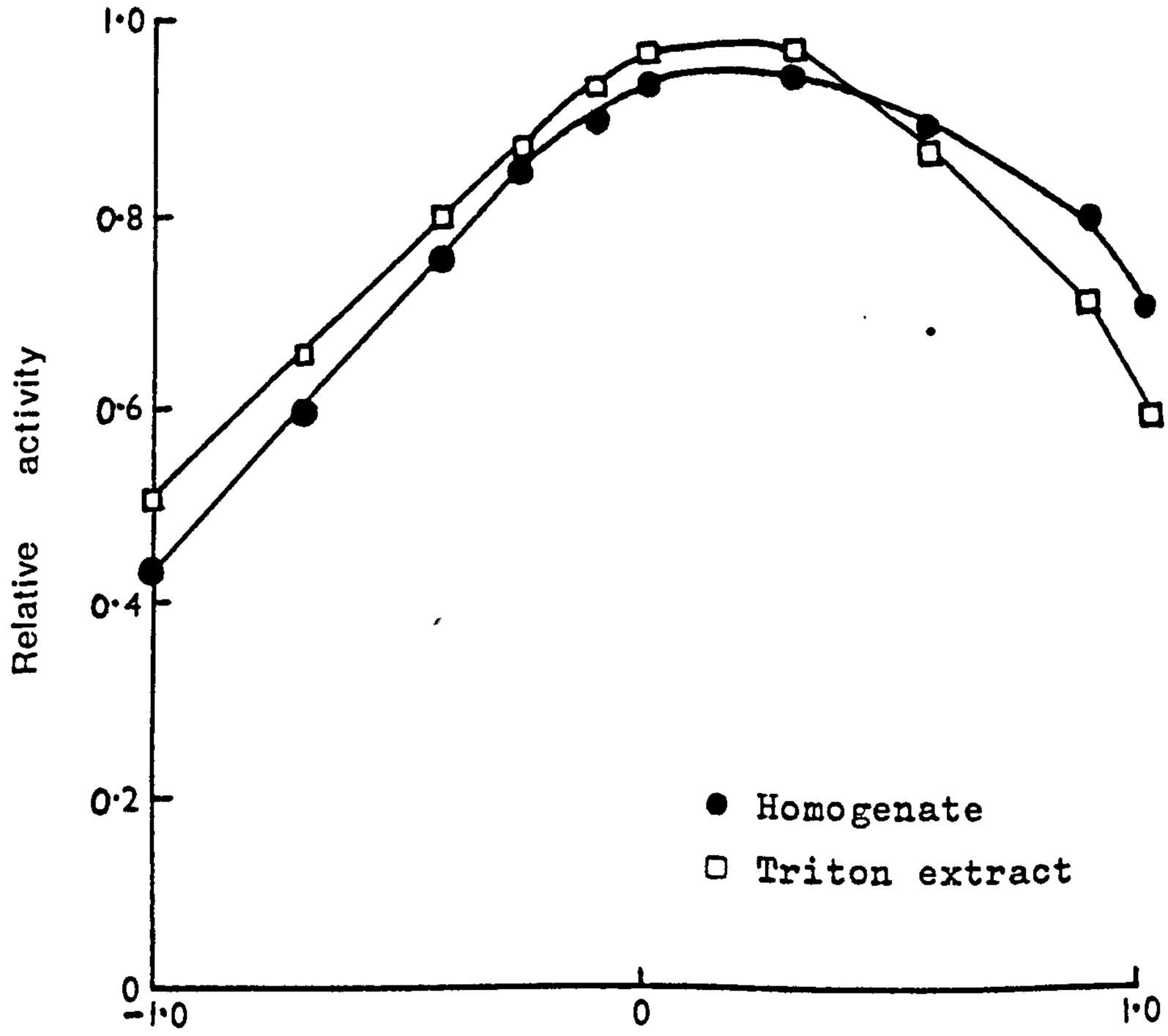


Fig. II.4 Substrate Dependence of Acetylcholinesterase in Rat Muscle



membrane-bound enzymes.

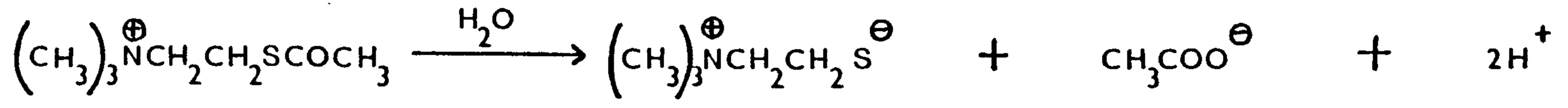
The substrate optima obtained for the homogenate preparations of both porcine brain and rat muscle was in the range 1 - 2 mmol/l, and for the solubilized enzyme it was 2 mmol/l. (Figs. II.2 and II.4). Therefore for standard assays a substrate concentration of 1 mmol/l was used.

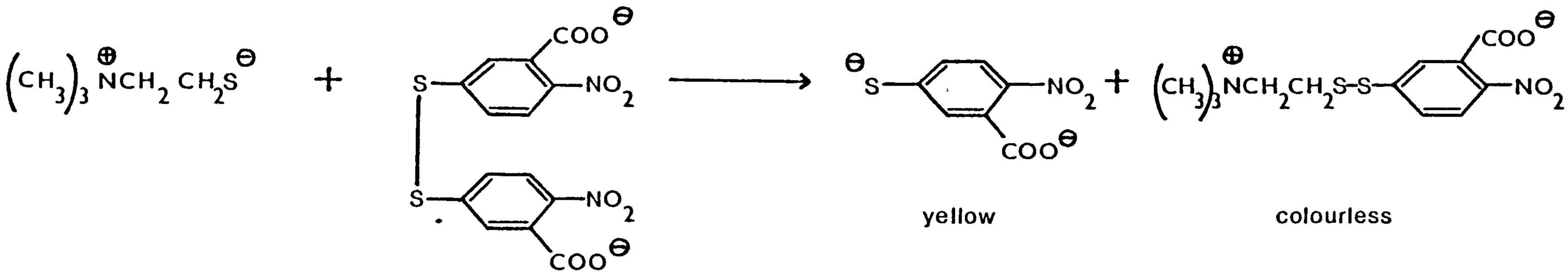
(ii) Ellman spectrophotometric method The Ellman method (Ellman et al, 1961) is confined to thioester substrates. The mercaptide formed from the hydrolysed thioester reacts with an oxidising agent, 5,5'-dithiobis-(2-nitrobenzoic acid),DTNB. The DTNB splits into two products one of which, 5-thio-2-nitrobenzoate, absorbs at 412 nm. Brownson and Watts, (1973) reported that the DTNB interacts with human erythrocyte AChE causing a marked activation, and so they recommended the use of 2,2'-dithiodipyridine as the oxidising agent. Augustinsson & Eriksson (1975) however disagree with these findings and advise the continued use of DTNB in the AChE assay. (Fig. II.5).

For routine assay, 50 µl. enzyme was added to 3 ml. sodium phosphate buffer (0.1 mol/l., pH 8.0) and incubated at 30°C. for 10 min. Then 100 µl. DTNB* (0.01 mol/l.) was added to the medium followed by 20 µl. acetylthiocholine iodide (158.5 mmol/l.) to give a final concentration of 1 mmol/l. substrate. The increase in absorbance was followed at 412 nm. on a Perkin-Elmer SP 124 double beam recording spectrophotometer.

* The stock DTNB was made up by dissolving 39.6 mg. in 10 ml. sodium phosphate buffer (0.1 mol/l., pH 7.0) containing 15 mg. sodium bicarbonate. The reagent is unstable at more alkaline pH's.

Fig.II.5 Ellman Colorimetric Reaction





DTNB

yellow

colourless

From the extinction coefficient of the chromophore which is 1.36×10^4 litre.mol^{-1}.cm^{-1}, the activity of the enzyme can be thus calculated:

$$\frac{\Delta E \times 1000 \times 3.17}{\text{min.} \times 1.36 \times 10^4 \times 0.05} = \frac{\Delta E}{\text{min.}} \times 4.66 \ \mu\text{mol.min}^{-1}\text{.ml}^{-1}$$

B. Protein

(i) Folin-Lowry method Protein was accurately determined by the method of Lowry et al, (1951) using crystalline bovine serum albumin as standard. However, if Triton X-100 was present, a gelatinous precipitate formed but this interference could be overcome by centrifuging the precipitate (1000 g, 5 min.) and incorporating Triton X-100 in the reagent blank and standards; (Hartree, 1972; Chandra Rajan & Klein, 1975).

(ii) Biuret method A modified biuret method was also used when measuring the protein content of a membrane suspension and in this case 1.5% (w/v) deoxycholic acid, sodium salt, was incorporated into the reagent to solubilize all the proteins present. The procedure for protein estimation was as follows: (a) 2 ml. of the biuret reagent was added to 1 ml. protein solution; (b) the mixture was heated at 100°C. in a boiling water-bath for 1 minute; (c) the mixture was cooled to room temperature and the extinction at 540 nm. was measured.

(iii) Ultraviolet absorption When many samples were being assayed, protein was estimated by measuring the absorbance at 280 nm.

providing Triton X-100 was absent. The detergent has a chromophore at 280 nm. which interferes with the readings and so when detergent was present a biuret method (Plummer, 1971) was sometimes used as an alternative.

2. METHODS OF SOLUBILIZATION

A. Porcine Brain Acetylcholinesterase

All solubilization procedures were performed on porcine brains obtained on the day of use from the Co-operative Society Slaughter House, Woolwich, London. The membranes and blood vessels were removed from the surface of the brains and the white matter excised and dispersed in a Waring Blender for 5 min. at 4°C with 0.03 mol/l sodium phosphate buffer (pH 7.0) to produce a 10% homogenate (g. wet weight per ml.). Centrifugations were carried out on MSE SS 65 or SS 50 preparative ultracentrifuges using 8 x 50 ml. or 10 x 10 ml. capacity rotors. The criterion of solubility was taken as the enzyme remaining in the supernatant after being centrifuged at 100,000 g. for 1 h.

(i) Aqueous media A 10% brain homogenate (g. wet weight per ml.) was prepared in 0.03 mol/l sodium phosphate buffer (pH 7.0) and this was centrifuged at 100,000 g. for 1 h. The supernatant was kept and is referred to as the 'naturally soluble' form of the enzyme.

(ii) Sodium deoxycholate The method of Marples *et al* (1959) and McArdle *et al* (1960) was followed, using sodium deoxycholate as the solubilizing agent. A 10% (w/v) suspension of white matter in sodium phosphate buffer (0.03 mol/l., pH 7.0) was incubated for 30 min. at 37°C with 0 - 0.5% (w/v) sodium deoxycholate. The extract was centrifuged at 100,000 g. for 1 h. and the supernatant taken as 'naturally soluble' enzyme. A control was taken through the same procedure but with the exclusion of sodium deoxycholate.

(iii) Sodium cholate Sodium cholate (0-1%, w/v) was used as a solubilizing agent, employing the same method as adopted for solubilization with sodium deoxycholate.

(iv) Lysolecithin treatment Marples et al (1959) and McArdle et al (1960) found that 12 mmol/l lysolecithin and an incubation time of 20 min. to 60 min. was optimal for solubilization of brain enzyme and these conditions were adopted. A 10% (w/v) suspension of white matter in sodium phosphate buffer (0.03 mol/l., pH 7.0) was incubated for 30 min. at 37°C with 12 mmol/l lysolecithin. The solution was centrifuged at 100,000 g. for 1 h. and the supernatant taken as 'naturally soluble' enzyme. A control was taken through the same procedure but with the exclusion of lysolecithin.

(v) Triton X-100 The method of Ho & Ellman (1969) was followed with slight modifications. A 10% (w/v) suspension of white matter in sodium phosphate buffer (0.03 mol/l., pH 7.0) was centrifuged at 100,000 g. for 1 h. The supernatant was decanted and this was taken to be the 'naturally soluble' enzyme. The pellet was resuspended to the original volume with buffer and Triton X-100 added to a final concentration of 0-2% (w/v). The mixture was stirred for 10 min. at room temperature and centrifuged at 100,000 g. for 1 h. The supernatant was taken to be the 'Triton solubilized' enzyme.

The use of a combination of Triton X-100 and potassium chloride has been shown by Wright and Plummer (1970,1972) to be more efficient than Triton X-100 alone in the solubilization of red blood cell AChE. A range of concentrations of detergent and KCl were therefore used in an attempt to see if this also applied to

the brain AChE.

A 10% (w/v) homogenate was treated with an equal volume of Triton X-100 (0-2%, w/v, final concentration) in the presence or absence of KCl (0-0.6 M final concentration). The period of shaking was increased to 40 min. since it was observed that an extension of the original 10 min. gave greater clarity to the suspension.

(vi) Chelating agent Solubilization with ethylene diamine tetra acetic acid (EDTA) was carried out by modifying the methods of Chan et al (1972b) and Hollunger & Niklasson (1973). A 10% (w/v) homogenate was prepared in sodium phosphate buffer (0.03 mol/l., pH 7.0) at 4^{o}C and centrifuged for 1 h. at 100,000 g. The pellet was resuspended to the original volume with buffer containing EDTA (1mmol/l.). The suspension was stirred for 2 h. at 4^{o}C and then centrifuged at 100,000 g. The supernatant was removed and the pellet treated a further two times in the same way.

B. Rat Muscle Acetylcholinesterase

Gastrocnemius muscles were excised from rats (strain did not matter) obtained from the college animal house. The solubilization procedure was performed on the muscles obtained on the day of use. Peripheral nerves were removed from the muscles and the muscles were cut into small pieces. A 20% muscle homogenate (g. wet weight per ml.) was prepared with 0.03 mol/l sodium phosphate buffer (pH 7.0) in a Waring Blender run for 5 min. at 4^{o}C. Centrifugations were carried out on MSE SS 65 or SS 50 preparative ultracentrifuges using 8 x 50 ml. or 10 x 10 ml. capacity rotors.

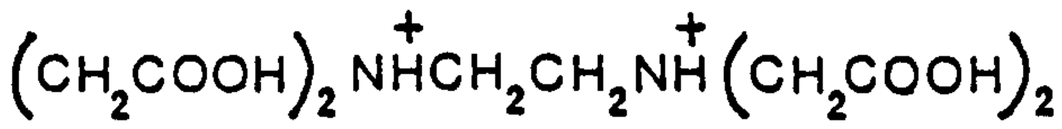
The criterion taken for the enzyme to be soluble was if it remained in the supernatant after centrifugation at 100,000 g. for 1 h.

(i) Aqueous media A 20% muscle homogenate (g. wet weight per ml.) was prepared in 0.03 mol/l sodium phosphate buffer (pH 7.0) and this was centrifuged at 100,000 g. for 1 h. The supernatant was kept and is referred to as the 'naturally soluble' enzyme.

(ii) Triton X-100 The method of Ho & Ellman (1969) was adopted with slight modifications. A 20% homogenate in sodium phosphate buffer, pH 7.0, was centrifuged at 100,000 g. for 1 h. The supernatant was decanted and is referred to as the 'naturally soluble' enzyme. The 100,000 g. pellet was resuspended to the original volume with sodium phosphate buffer (0.03 mol/l, pH 7.0) incorporated with Triton X-100 (0-2%, w/v, final concentration). The mixture was stirred for 10 min. at room temperature and then centrifuged at 100,000 g. for 1 h. The supernatant was taken to be the 'Triton solubilized' enzyme.

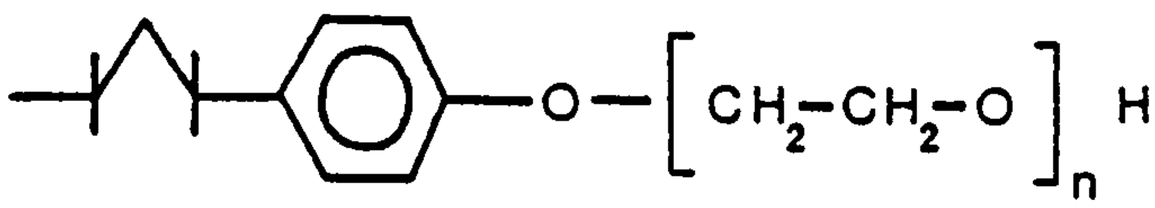
Fig. II.6 shows the formulae of the solubilizing agents used.

Fig. II.6 Solubilizing Agents



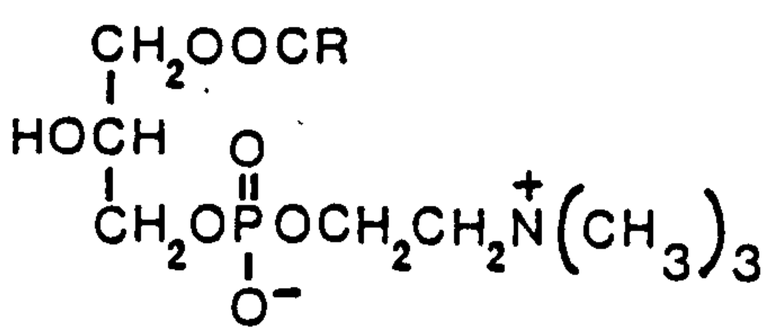
Ethylenediaminetetraacetic acid (EDTA)

(Ethane, 1, 2-diamino-N,N,N',N'-tetraacetic acid)



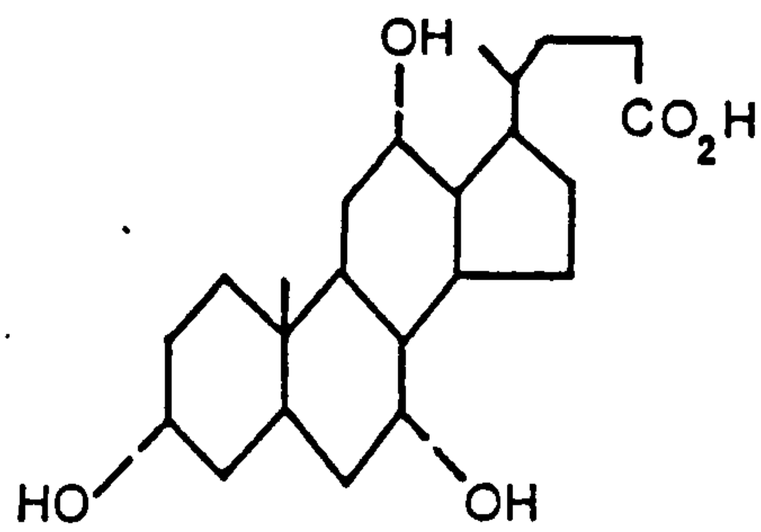
Triton X-100

(p-isooctylphenoxypolyethoxyethanol)



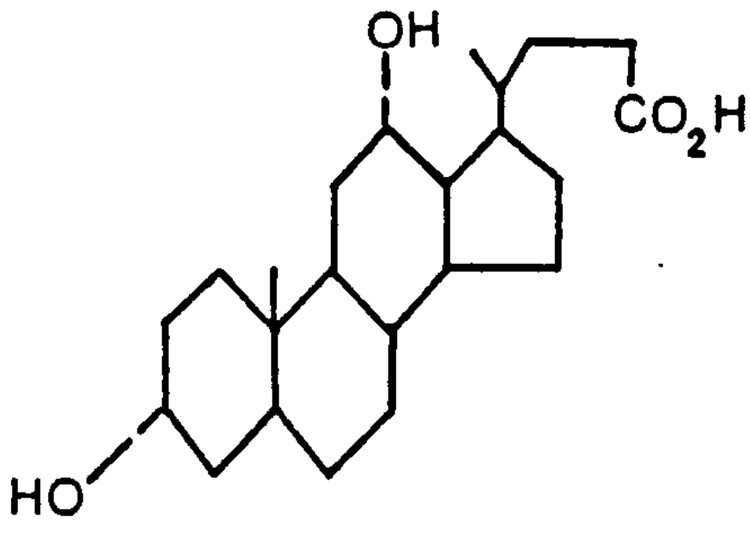
Lysolecithin

(Lysophosphatidylcholine)



Cholic Acid

(3α, 7α, 12α-trihydroxy-cholanic acid)



Deoxycholic Acid

(3α, 12α-dihydroxy-cholanic acid)

3. AFFINITY CHROMATOGRAPHY

Affinity column synthesis fell into two stages. Firstly, a beaded agarose resin was activated under stringent conditions of pH and temperature. Secondly, an active site ligand specific for AChE was bound to the resin matrix via a spacer arm. For the MAC-agarose column, the ligand and spacer arm were synthesised '*in toto*' and then coupled to the activated agarose.

A. Materials

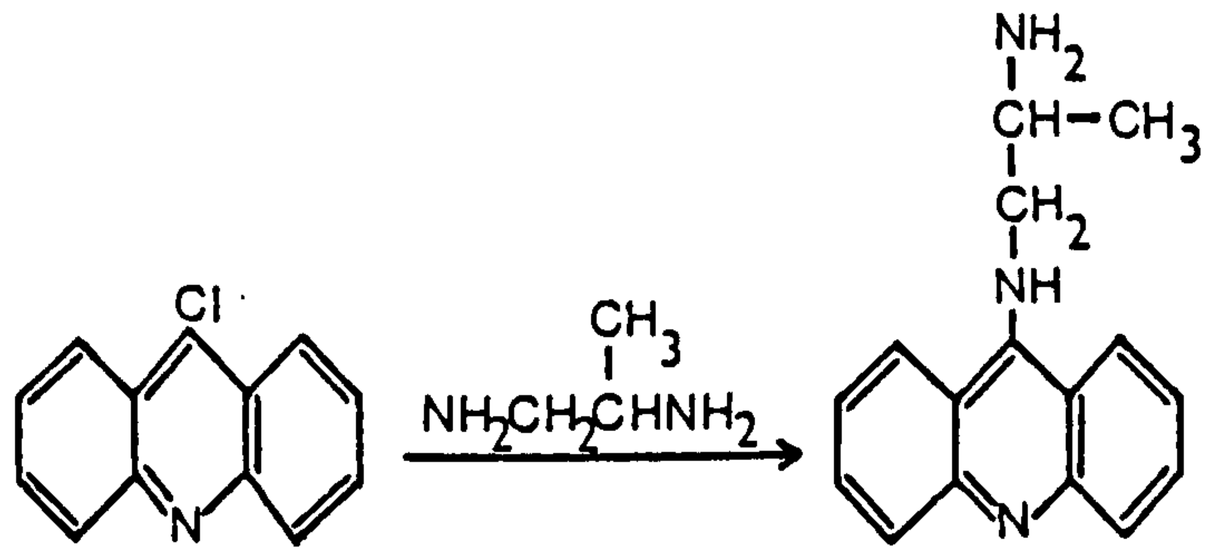
Materials for the MAC-agarose column were obtained from the following sources. N-Benzyloxycarbonyl-ε-aminocaproic acid was purchased from Aldrich Chemical Company Ltd., Wembley, Middlesex. Triethylamine was supplied by Fisons, Loughborough, Leics. Anhydrous HBr in glacial acetic acid, isobutylchloroformate, iodomethane and 9-chloroacridine were obtained from Eastman Kodak Co., Rochester, N.Y., U.S.A. Phenol was obtained from Fisons and redistilled before use.

B. MAC-Agarose Column

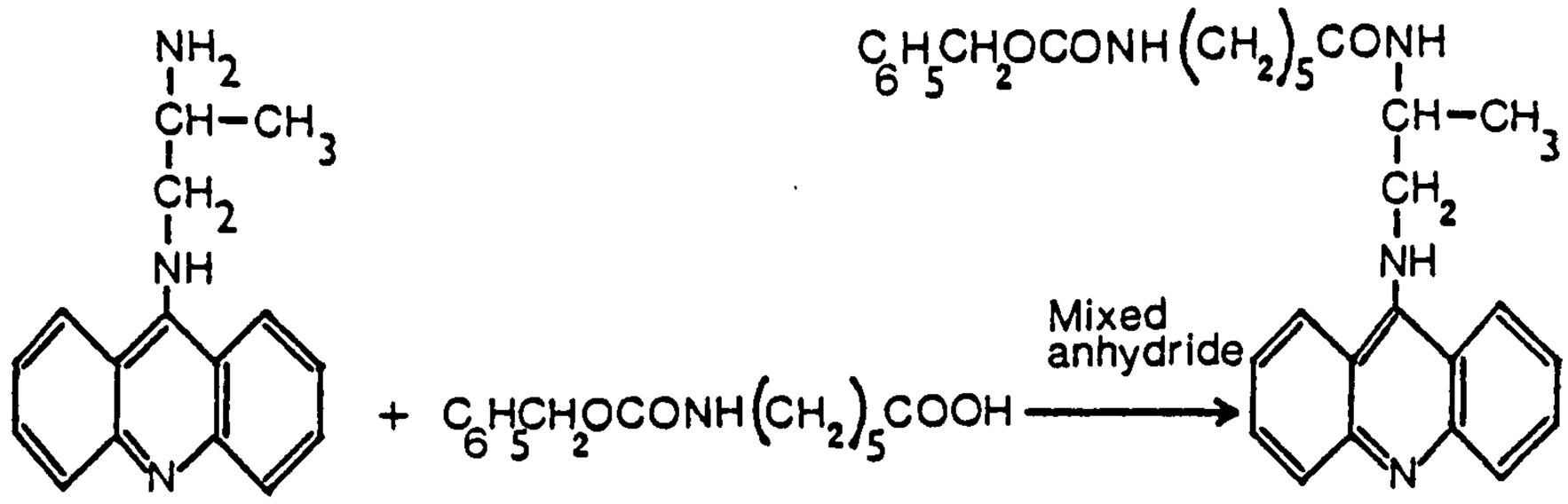
(i) Preparation of the ligand (1-methyl-9-[N^{β}-ε-aminocaproyl)-β-aminopropylamino] acridinium bromide hydrobromide

The ligand was synthesised by the method of Dudai & Silman (1974a) with some amendments advised by Silman (personal communication). See Fig. II.7. Phenol (1.28 mol; 120 g.) was heated to 70°C. and 9-chloroacridine (93.60 mmol; 20 g.) added to the melt. After all the solid had dissolved 100 ml. 1,2-propylenediamine was added to

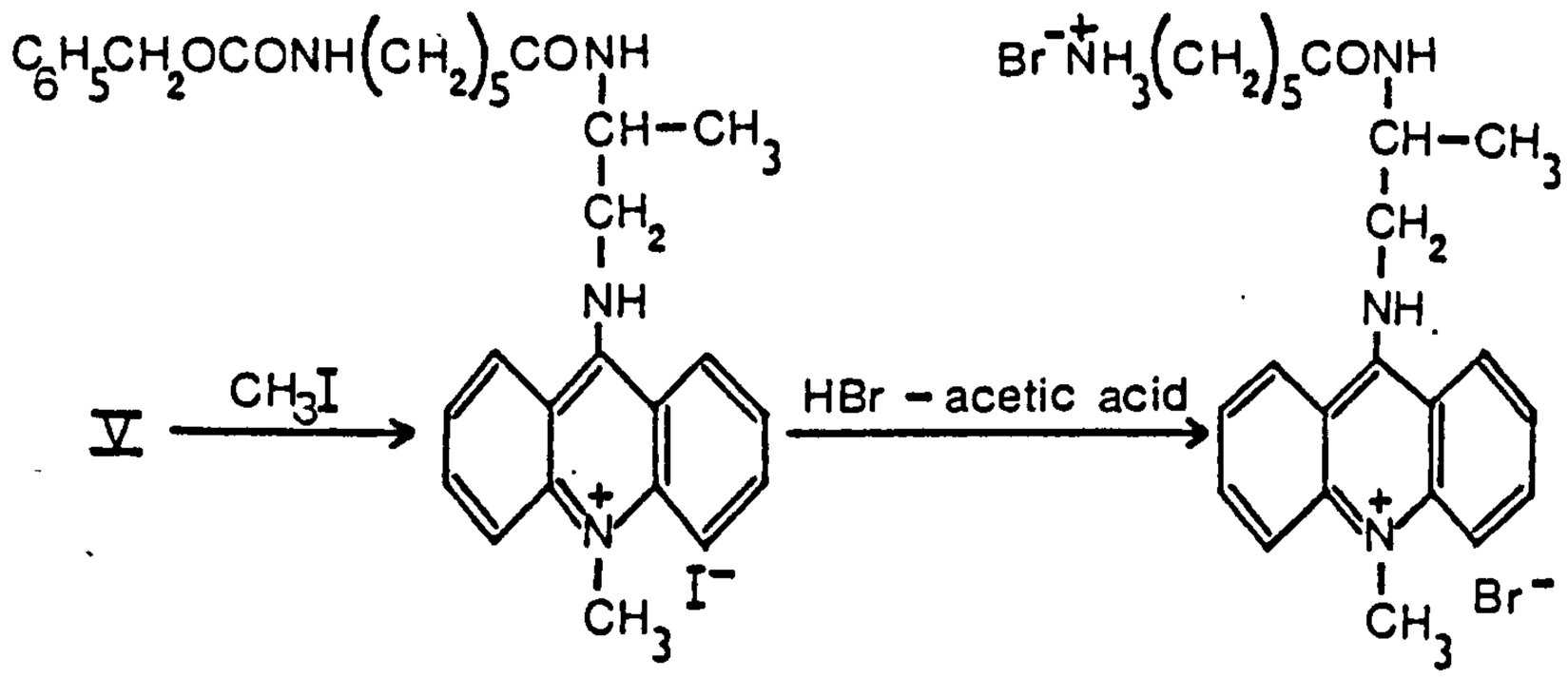
Fig. II.7 Synthesis of 1-methyl-9-[N^{β}-(ξ-aminocaproyl)-β-aminopropylamino]-acridinium bromide hydrobromide, (VII), MAC



IV



V



VI

VII

MAC

the vigorously stirred mixture and the temperature raised to 120°C. It was critical that this temperature was not exceeded. After 30 min. the product 9-(β-aminopropylamino) acridine (compound IV) was precipitated by pouring the reaction mixture with rapid stirring into 1600 ml. of NaOH (0.75 mol/l.). This was left overnight to complete the solidification, filtered and washed with NaOH (2 mol/l.) followed by water. The crude product was dried in vacuo then refluxed in 1100 ml. absolute benzene and filtered. The insoluble residue was discarded and the filtrate concentrated to 200 ml. Then 50 ml. petroleum was added to the filtrate and left at 4°C. overnight. The crystalline product was filtered and washed with benzene.

Yield of 9-(β-aminopropylamino) acridine (compound IV) =

9.10 g. (38.2%)

m.p. found = 131-138°C.

m.p. lit. = 131-133°C.

The mixed anhydride method of Greenstein & Winitz (1961) was employed for the next step. N-Benzyloxycarbonyl-ε-aminocaproic acid (30 mmol; 7.9 g.) was dissolved in 150 ml. dry ethyl acetate in a salt-ice bath at -10°C. and stirred vigorously. To this mixture was added triethylamine (30 mmol; 3.05 g.) followed by isobutylchloroformate (30 mmol; 4.1 g.) and this was left stirring for 20 min. at -10°C. The reaction mixture was filtered by suction and the precipitated triethylamine hydrochloride washed with 20 ml. dry ethyl acetate. The combined filtrate and washings was returned to the salt-ice bath and a solution of 9-(β-aminopropylamino) acridine (25 mmol; 6.3 g.) dissolved in dry dimethylformamide (100 ml.) at -10°C. was added. This was left for a further

20 min. at this temperature and then for 12 h. at room temperature. Thin layer chromatography on silica gel in glacial acetic acid indicated that there had been total coupling to give compound V. The reaction mixture was evaporated to dryness under reduced pressure and the residue dissolved in 50 ml. absolute methanol. Then 8 ml. iodomethane was added to the solution, refluxed for 4 h. and left overnight at room temperature. Silica gel thin layer chromatography in ethyl acetate showed that quaternization had gone to completion. The solution was evaporated dry under reduced pressure, extracted twice with 100 ml. aliquots of dry ethyl acetate and the product recrystallized from 80 ml. 2-propanol. The solid compound VI, [N-(N-benzyloxycarbonyl-ε-aminocaproyl)-β-aminopropylamino] acridinium iodide was washed with ice-cold 2-propanol followed by ice-cold diethyl ether.

Yield of compound VI = 6.1 g.
m.p. found = 154.5-157.5°C.
m.p. lit. = 156-157°C.

The above quaternary compound VI (6.4 mmol; 4.1 g.) was dissolved in 40 ml. anhydrous glacial acetic acid and then 80 ml. anhydrous HBr in glacial acetic acid was added. This solution was left for 30 min. at room temperature and the product precipitated with dry diethyl ether. The precipitate was triturated with 5 batches of diethyl ether until it solidified. The solid was filtered, washed with diethyl ether and left for 24 h. under reduced pressure over dry NaOH pellets. The resulting crystals of (1-methyl-9-[N^{β}-ε-aminocaproyl)-β-aminopropylamino] acridinium bromide hydrobromide, (MAC) were recrystallized from absolute ethanol.

Yield of MAC = 2.1 g. (60.5%)
m.p. found = 235-240°C.
m.p. lit = >240°C. with decomposition

(ii) Preparation of MAC-Agarose The ligand was coupled to the Sepharose 4B according to the method of Axen et al (1967) as modified by Blumberg et al (1970). Cyanogen bromide (7 g.) was added to 70 ml. water and stirred for 10 min. during which time most of it dissolved. A Sepharose 4B slurry was washed with water and 70 ml. of this added to the stirred cyanogen bromide-water mixture. The pH was immediately adjusted to 11 with 6 mol/l-NaOH and the mixture cooled below 20°C. with crushed ice for 8 min. The activated gel was then rapidly washed with 1000 ml. water at 4°C. on a Buchner funnel and 70 ml. of the wet Sepharose 4B added quickly to MAC (0.09 mmol; 50 mg.). This mixture was shaken gently (not mechanically stirred) for 16 h. at 4°C., then filtered and washed thoroughly with $NaHCO_3$ (0.1 mol/l.) and water. The amount of ligand coupled was found by estimating spectrophotometrically the quantity of ligand remaining in the washings. Approximately 0.5-1.0 μmol MAC were found to be coupled per ml. resin.

At pH 8.3, MAC λ max = 393 nm; 410 nm; 431 nm;
ε = 7,880; 12,050; 10,150;

The affinity resin was washed extensively before use with the elution buffer.

C. Conditions for Elution

The buffer used for elution was sodium phosphate buffer (0.03 mol/l., pH 7.0) which was run through columns with a bed

volume of 12 ml. The enzyme was run through the resin at a rate of 25-35 ml/h. after which the column was washed with 5-10 column volumes of buffer. The enzyme was then eluted with 5 column volumes of elution buffer containing the AChE inhibitor decamethonium bromide (10 mmol/l.) and fractions of 2-5 ml. collected. Finally the column was washed with 5 column volumes of buffer containing NaCl (1 mol/l.) followed by guanidine hydrochloride (6 mol/l.) and then 50 column volumes elution buffer. The peak of enzyme activity was dialysed against three changes of 2000 ml. elution buffer over 72 h. All procedures were conducted at 4°C. Fig. II.8 shows the MAC-agarose ligand.

The above elution programme was essentially based on procedures followed by Dudai et al (1972a); Dudai et al (1972b); Berman & Young (1971); Goodkin & Howard (1974); with extensive modifications.

Fig. II.8 MAC-agarose

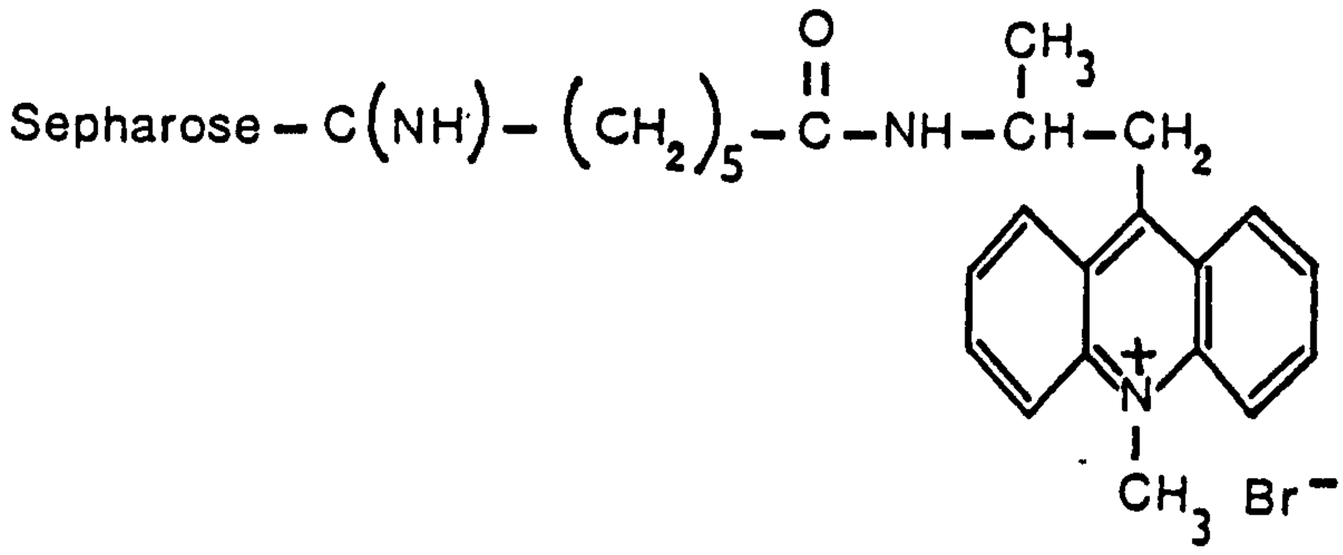
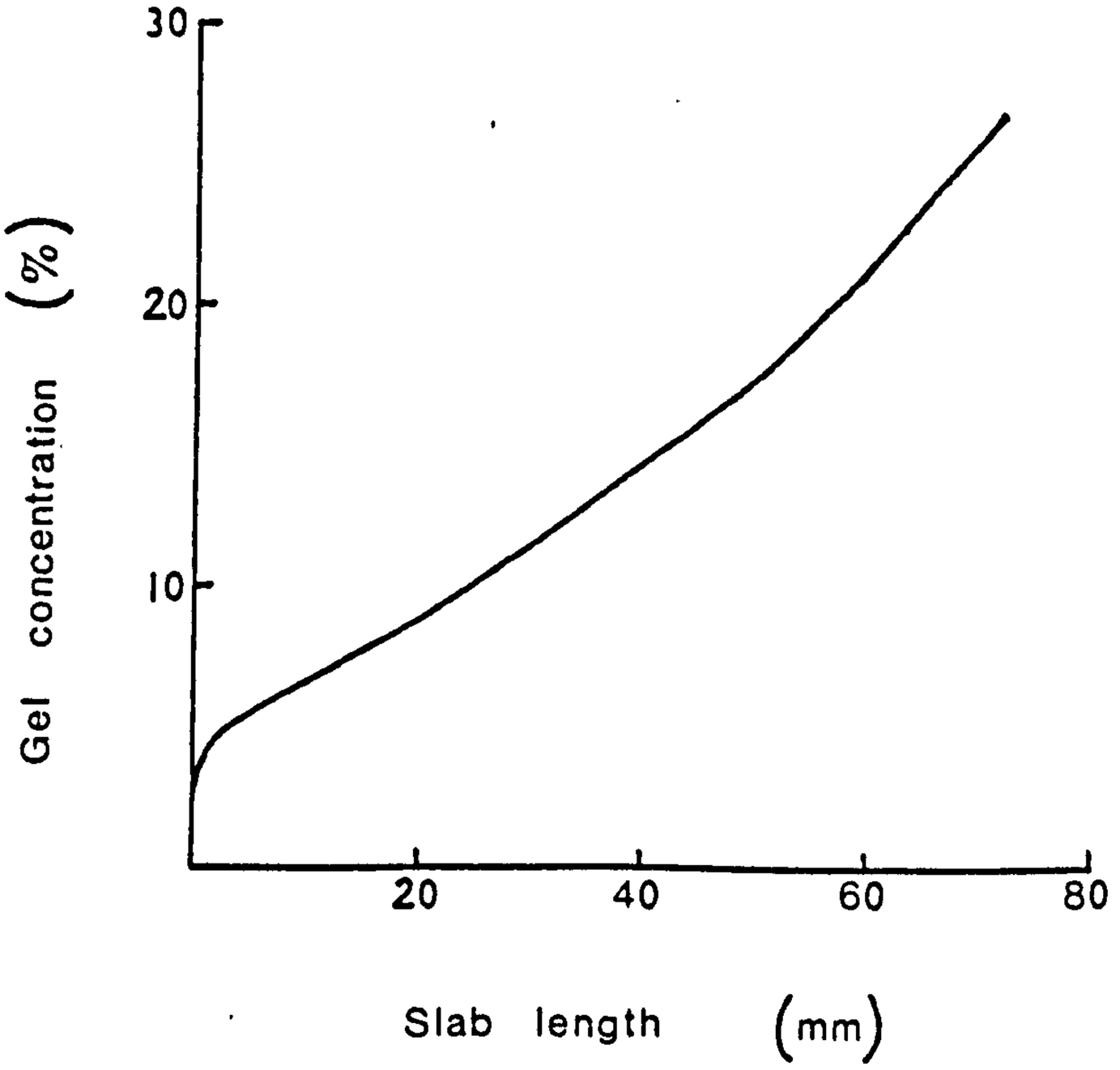


Fig. II.9 Gel Concentration Plotted Against Slab Length of Polyacrylamide Gradient Gels



4. ELECTROPHORESIS

A. Polyacrylamide Rods

Ornstein, (1964); Davis, (1964).

Stock solutions were prepared as follows:

Solution A

1 mol/l - HCl	48 ml.
Tris	36.6 g.
TEMED	0.23 ml.
Water to	100 ml.
pH 8.9	

Solution B

1 mol/l - HCl	48 ml.
Tris	5.98 g.
TEMED	0.46 ml.
Water to	100 ml.
pH 6.9	
Adjust with 1 mol/l - HCl	

Solution C

Acrylamide	28.0 g.
Bis	0.735 g.
Water to	100 ml.

Solution D

Acrylamide	10.0 g.
Bis	2.5 g.
Water to	100 ml.

Solution E

Riboflavin	4 mg.
Water to	100 ml.

Solution F

Sucrose	40 g.
Water to	100 ml.

Working Solutions

Small pore solution 1

1 part A
2 parts C
1 part water

Small pore solution 2

Ammonium persulphate 0.14 g.
Water to 100 ml.

Large pore solution

1 part B
2 parts D
1 part E
4 parts F
pH 6.7

Stock buffer solution

Tris	6.0 g.
Glycine	28.8 g.
Water to	1 litre
pH 8.3	

Equal volumes of small pore solutions 1 and 2 were mixed, degassed and aliquots of 0.9 ml. added to eight electrophoresis

tubes. A water overlay was applied to the surface of the solutions and these were left to polymerise (20-40 min.). The water overlays were removed and 0.15 ml. degassed large pore solution syringed on top of the gels. The solutions were overlaid with water and the tubes placed in a fluorescent light until polymerisation was complete (20-40 min.). A mixture of 150 µl. large pore solution and 10-40 µl. sample protein was added to each tube and polymerised as described above.

The stock electrophoresis buffer was diluted 10-fold with water and poured in the anodic and cathodic reservoirs of a Quickfit PAGE apparatus. Eight gels were run per apparatus at a constant current of 2.5 mA per tube for 1½-3 h. at 4°C.

Protein was stained by placing the gels in Coomassie Brilliant Blue Stain (0.2% w/v) in methanol : acetic acid : water in the ratios 5 : 1 : 5 for 24 h. The gels were incubated in acetic acid (7% v/v) for periods long enough to remove the background stain.

AChE was stained by the method of Koelle (1951) as modified by Lewis & Shute (1966). Acetylthiocholine iodide (100 mg.) was dissolved in 8 ml. water and 14 ml. cupric acetate was added dropwise to the stirred solution. This was centrifuged at 2000 rpm. for 10 min. and the supernatant decanted into a beaker containing glycine (60 mg.). Sodium acetate (2 mol/l.) was added to the solution to give a final pH between 6.5 and 7.0. Gels were incubated in this stain for 6-24 h. and destained in 7% v/v acetic acid.

B. Polyacrylamide Slabs

'Gradipore' polyacrylamide gradient gels (4-24%) purchased from Universal Scientific Ltd., London, were used (Fig. II.9). The buffer used was Tris-borate-EDTA buffer solution (pH 8.3) and it was made up as follows:

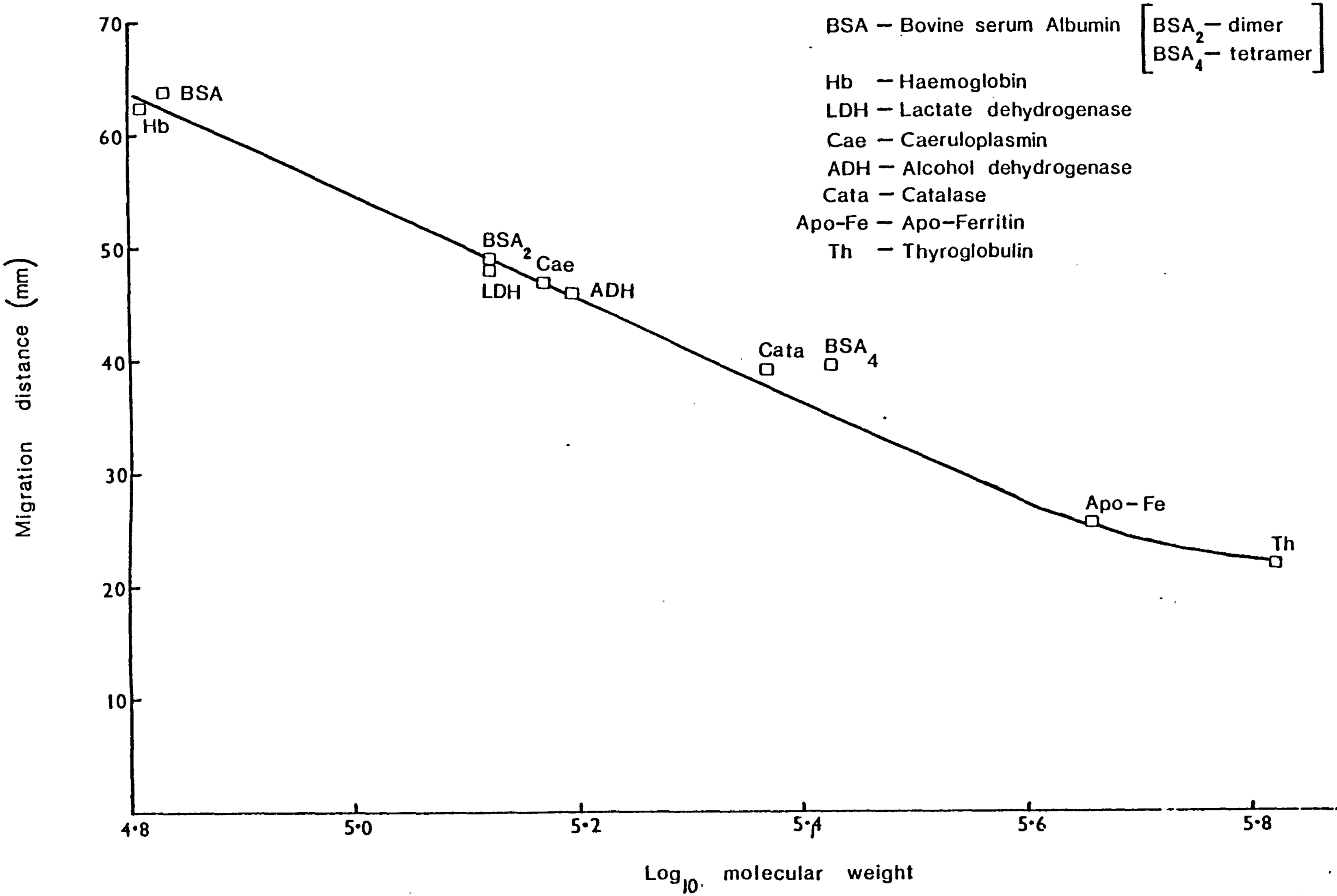
EDTA.	4.65 g.	2.5 mmol/l.
Tris	53.75 g.	88.74 mmol/l.
Boric acid	25.20 g.	81.51 mmol/l.
Water to	5 l.	

Gels were pre-electrophoresed without samples in a three cell 'Gradipore' electrophoresis unit at 100 v. until the current had fallen to a steady value between 30-35 mA. Samples (40 μl.) were applied to the top of the gels in a plastic spacer which allowed 14 samples to be run per gel. The samples were overlaid with buffer and electrophoresis carried out for 24 h. at 100 v. and room temperature. The buffer was circulated through a heat exchanger immersed in a water bath to keep the temperature stable.

Staining of the gels for protein and enzyme was accomplished by removing them from the glass retaining plates and treating them in the same way as polyacrylamide rods.

Each batch of gels was standardized by electrophoresing several proteins of known molecular weight and plotting migration distance against $\log_{10}$ molecular weight. The proteins used were haemoglobin, lactate dehydrogenase, alcohol dehydrogenase, bovine serum albumin, caeruloplasmin, horse spleen apoferritin and thyroglobulin. (Fig. II.10).

Fig. II.10 Calibration Curve for Polyacrylamide Gradient Gels



C. Starch Block Electrophoresis

This method, though too laborious as a routine analytical tool has its use as a semi-preparative procedure.

Potato starch was obtained from British Drug House, Poole, Dorset. The starch was washed twice with water and then twice with sodium phosphate buffer (0.1 mol/l., pH 8.0). Triton X-100 (1% w/v) was incorporated in the buffer if the sample to be electrophoresed contained the detergent. Excess buffer was decanted and the starch pressed into a block with the aid of perspex formers 19 cm. X 12 cm. X 1 cm. or 30 cm. X 10 cm. X 1 cm. Wicks consisting of several layers of muslin connected the block with the buffer in the electrode tanks.

Anode and cathode tanks were each divided into two sections connected by cotton wool plugs. This arrangement prevented pH changes around the electrodes affecting the buffer in contact with the block. The block was pre-electrophoresed at 6-7 v/cm. for 1 h. at 4°C. The enzyme sample containing 5-10 mg. protein was applied to a laterally cut groove, 0.5 cm. wide and one-third of the distance along the block from the cathode. A mixture of haemaglobin, bovine serum albumin and bromophenol blue was applied as markers to a cavity in line with the groove. The cavity and groove were filled with starch and electrophoresis conducted at 6-7 v/cm. for times up to 18 h.

The final positions of markers were noted and the longitudinal section containing them discarded. The remaining block was cut transversely into strips of 0.5 cm. width between the markers and 1 cm. width either side of the markers. Each portion of starch was eluted twice through a sintered glass funnel with 2 ml.

aliquots of buffer. The AChE was assayed by the Ellman method and the electrophoresis patterns represented by histograms showing total activity in each strip cut from the block.

5. DENSITY GRADIENT CENTRIFUGATION

Sucrose gradients, used in the swing-out rotors of a preparative ultracentrifuge, were employed to determine the sedimentation coefficient and approximate molecular weight of AChE. This was possible by determining the ratio of mobilities between AChE and a standard well characterized protein (Martin & Ames, 1961).

A. Preparation of Gradients

Sucrose gradients were prepared in 5 ml. capacity polycarbonate tubes. Sucrose was added in layers of equal volume and of diminishing concentration. The bottom layer of sucrose was 60% w/v and on top of this was pipetted ten equal volumes (0.4 ml.) of buffered sucrose starting with 20% w/v diminishing finally to 5% w/v. The tube was then rotated gently back and forth around its longitudinal axis to disperse the interfaces and thus obtain a homogeneous linear gradient. Gradients were stored at $4^{o}C$ for 5 h. before use.

B. Centrifugation

Bovine catalase, which was assumed to have a sedimentation coefficient of 11.4 S. and mol. wt. 240,000 (Sumner & Gralén, 1938), was mixed with the enzyme sample and layered by pipette on to the gradients. It was important that not more than 50 mg. protein was applied to each gradient as overloading causes loss of Gaussian shape of the migrating protein zone (Steensgaard et al, 1975).

After spinning at 100,000 g. for 17 h., the tubes were fractionated and aliquots removed with an MSE tube piercer. Fractions consisting of 3 drops from the 5 ml. tubes were collected and then assayed for AChE by the Ellman method. Catalase was assayed by following the decrease in absorbance at 240 nm. of a mixture containing 3 ml. sodium phosphate buffer (10 mmol/l., pH 7.5), 20 μl. hydrogen peroxide (0.9 mol/l.) and 20 μl. enzyme sample. Activities were calculated in terms of change in absorbance per minute.

The ratio 'R' was determined experimentally according to Martin & Ames (1961):

$$R = \frac{\text{distance travelled from meniscus by unknown}}{\text{distance travelled from meniscus by standard}}$$

As molecules move at an almost uniform rate:

$$R = \frac{s^{0.725}_{20,w} \text{ unknown}}{s^{0.725}_{20,w} \text{ standard}}$$

where $s^{0.725}_{20,w}$ = sedimentation constant extrapolated to the standard state taken as that of water and partial specific volume 0.725 cm.^{3} g^{-1}. As most proteins have partial specific volumes in the range 0.70-0.75 cm.^{3} g^{-1}, the above assumption resulted in less than 3% error in the estimation of $S_{20,w}$.

So, for molecules of the same partial specific volume:

$$R = \frac{S_{20,w} \text{ unknown}}{S_{20,w} \text{ standard}}$$

A crude estimation of mol. wt. was obtained:

$$\frac{S_1}{S_2} = \left(\frac{\text{Mol. wt.}_1}{\text{Mol. wt.}_2}\right)^{\frac{2}{3}}$$

Since for most proteins S_1/S_2 is equal to R. (Schachman, 1959).

In order to test the linearity of the gradients, some centrifugations were performed in the absence of samples. The sucrose concentrations were then determined by a refractometer and plotted graphically against fraction number.

In addition, the efficacy of this technique was tested by running three standard proteins on gradients and comparing their sedimentation coefficients with those found by other techniques. The proteins used were yeast alcohol dehydrogenase, egg white muramidase and beef liver catalase. The assays were performed according to Martin & Ames (1961) and sedimentation values were assumed to be those quoted by Martin & Ames: catalase, 11.4 S; muramidase, 2.1 S; alcohol dehydrogenase, 7.6 S.

6. INHIBITION BY ORGANOPHOSPHORUS COMPOUNDS

The inhibition of AChE by a range of organophosphorus compounds was carried out both in the presence and in the absence of the substrate acetylthiocholine iodide. The organophosphorus compounds used were malaoxon, ethyl malaoxon, paraoxon, isopropyl paraoxon and methyl paraoxon.* Acetylcholinesterase activity was measured at 25°C and pH 7.5 by the technique of Ellman et al (1961) with acetylthiocholine iodide as substrate. The final concentrations of substrate and 5,5'-dithiobis-(2-nitrobenzoic acid) in the assay mixture were 1 mmol/l.

A. Inhibition in the Absence of Substrate

In the absence of substrate, the final concentration of inhibitor used was from 1 umol/l to 10 umol/l. Solutions of the inhibitor (500 µl) and enzyme (500 µl) in buffer (0.03 mol/l phosphate buffer, pH 7.5) were mixed at 25°C, and 100 µl portions added at intervals of 30 s. to cuvettes containing 2.9 ml. of substrate plus 5,5'-dithiobis-(2-nitrobenzoic acid). The absorbance at 412 nm. was recorded for 2-5 min. With enzymes prepared in the presence of Triton X-100, activity was constant during the assay because the 30-fold dilution with substrate prevented further inhibition, and there was no reactivation of inhibited enzyme. However, in the absence of detergent, inhibition continued after dilution and residual enzyme activity at the time of dilution was estimated from the initial slope of the plot of absorbance against time. Pseudo-first-order constants and bimolecular rate constants were calculated as described by Main &

Iverson (1966) and their accuracies computed by the least-squares method of linear regression with a maximum-likelihood programme.

B. Inhibition in the Presence of Substrate

In the presence of substrate, the final concentration of inhibitor used was from 10 μmol/l to 100 μmol/l. Enzyme (1 ml.) was added to substrate (1.5 mmol/l) and 5,5'-dithiobis-(2-nitro-benzoic acid) (1.5 mmol/l) in buffer (2 ml.) containing various concentrations of inhibitor, and the absorbance was recorded for 1-3 min. The resulting curves, analysed by using the maximum-likelihood programme, had first-order kinetics. The first-order rate constants were used to calculate the bimolecular rate constants, correcting for the presence of substrate as described by Hart & O'Brien (1973).

* Trivial names of organophosphorus compounds:

Malaoxon {diethyl [(dimethoxyphosphinyl)-thio] butanedioate} and its OO-diethyl homologue.

Paraoxon (diethyl 4-nitrophenylphosphate) and its dimethyl and di-isopropyl homologues.

The above compounds were analytical standards whose identity and purity were confirmed by n.m.r. (nuclear-magnetic-resonance) spectrometry and g.l.c..

Malaoxon was donated by Dr. W. Welling, Laboratorium voor Insekticidenonderzoek, Wageningen, The Netherlands, and the other compounds were donated by Bayer A.G., 509 Leverkusen, Bayerwerk, West Germany.

SECTION III

RESULTS

ACETYLCHOLINESTERASE FROM THE WHITE MATTER OF PORCINE BRAIN

1. SOLUBILIZATION AND PURIFICATION

A. Dilute Buffer or Aqueous Media

The criterion taken for the enzyme to be soluble was if it remained in the supernatant after centrifugation at 100,000 g. for 1 h.

When a 10% homogenate (g. wet weight per ml.) of white matter was extracted with water or 0.03 mol/l sodium phosphate buffer (pH 7.0), 15% of the AChE was soluble (range, 13% - 17%). This fraction was referred to as the 'naturally soluble' enzyme. Subsequent extraction of the pellet did not bring any more enzyme into solution.

The remaining AChE activity was sedimented after centrifugation at 100,000 g. for 1 h. The pellet was resuspended in buffer and assayed for enzyme activity. It was found that 84% (range, 83% - 85%) of the total enzyme activity of the homogenate was present in the 100,000 g. pellet and this represented the membrane-bound form of the enzyme (Table III.1).

Attempts were therefore made to bring this membrane-bound AChE into solution by extraction with chelating agent such as EDTA, with detergent such as Triton X-100, with lysolecithin, and finally, with bile salts such as sodium cholate and sodium deoxycholate. In the following text, the solubilization of AChE from the membrane fraction refers to the extraction of the enzyme from the 100,000 g. pellet, after the naturally soluble AChE had been solubilized.

Table III.1

Acetylcholinesterase in the Sodium Deoxycholate solubilized Fraction

Fraction	Activity/g. wet wt. u/g. wet wt.	Yield of activity %	Protein mg/g. wet wt.	Yield of protein %	Specific activity u/mg. protein
Homogenate	3.80 ± 0.14	100	79.2 ± 2.5	100	0.048
100,000 g. pellet	3.19 ± 0.15	83 - 85	58.6 ± 3.3	70.4-77.6	0.054
100,000 g. supernatant from sodium deoxycholate treatment of resuspended pellet	2.60 ± 0.13	66 - 72	41.9 ± 1.6	50 - 56	0.062

The results show the mean value ± S.E.M. for 20 experiments.

Units of enzyme activity (u) are μmoles acetylthiocholine iodide hydrolysed per minute.

B. Ethylene diaminetetra acetic acid (EDTA)

Repeated extraction of AChE from the 100,000 g. pellet with EDTA (1 mmol/l) brought a small amount of enzyme into solution. This fraction represented 3% (range, 2.8% - 3.2%) of the total homogenate activity. The yield was not improved by altering the conditions of the extraction procedure such as decreasing the percentage w/v of brain homogenate.

C. Triton X-100

The non-ionic detergent, Triton X-100 was not very effective as a solubilizing agent. Extraction of the enzyme from the 100,000 g. pellet with 1% (w/v) Triton X-100 solubilized 17% (range, 16% - 18%) of the total homogenate activity. As Triton X-100 was added in increasing concentration (0.1% - 2%, w/v), the amount of AChE solubilized increased from 16% to 18% of the total homogenate activity (Fig. III.1).

The addition of KCl to Triton X-100 in the extraction procedure affected the yield of AChE. The use of a combination of Triton X-100 and KCl at a concentration of 1% Triton X-100 plus 0-0.3 mol/l KCl, marginally increased the yield of AChE from 17% to 20% of the total homogenate activity. When 1% Triton X-100 plus 0.6 mol/l KCl was included in the extraction buffer, the enzyme activity decreased to 17% of the total homogenate activity. (Fig. III.2)

D. Lysolecithin

Lysolecithin was a more efficient solubilizing agent than EDTA or Triton X-100. When lysolecithin (12 mmol/l) was incorporated into the extraction buffer, 33% (range, 30% - 36%) of the total homogenate activity was solubilized.

Fig. III.1 Effect of Triton X-100 on Solubilization of Acetylcholinesterase

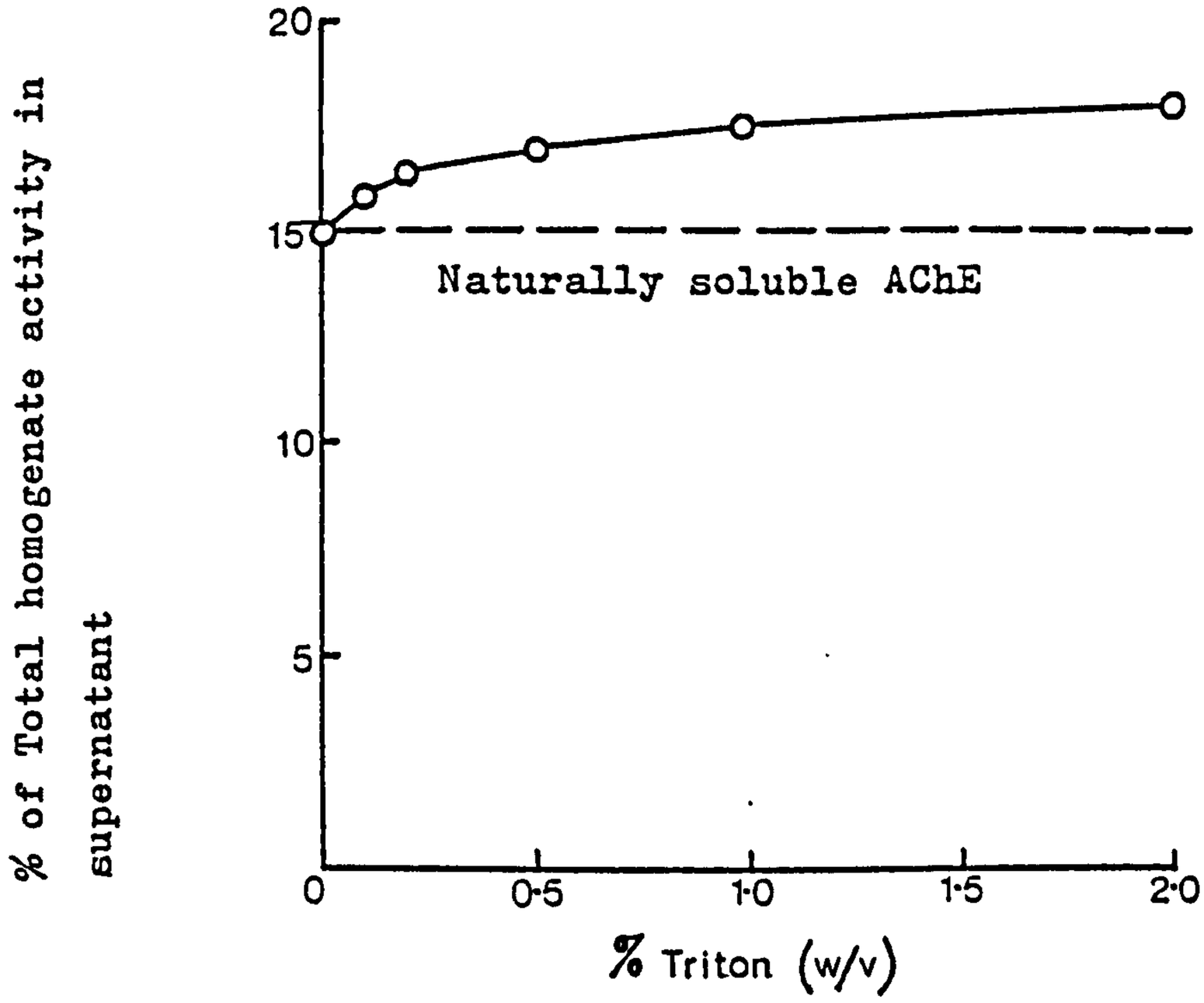
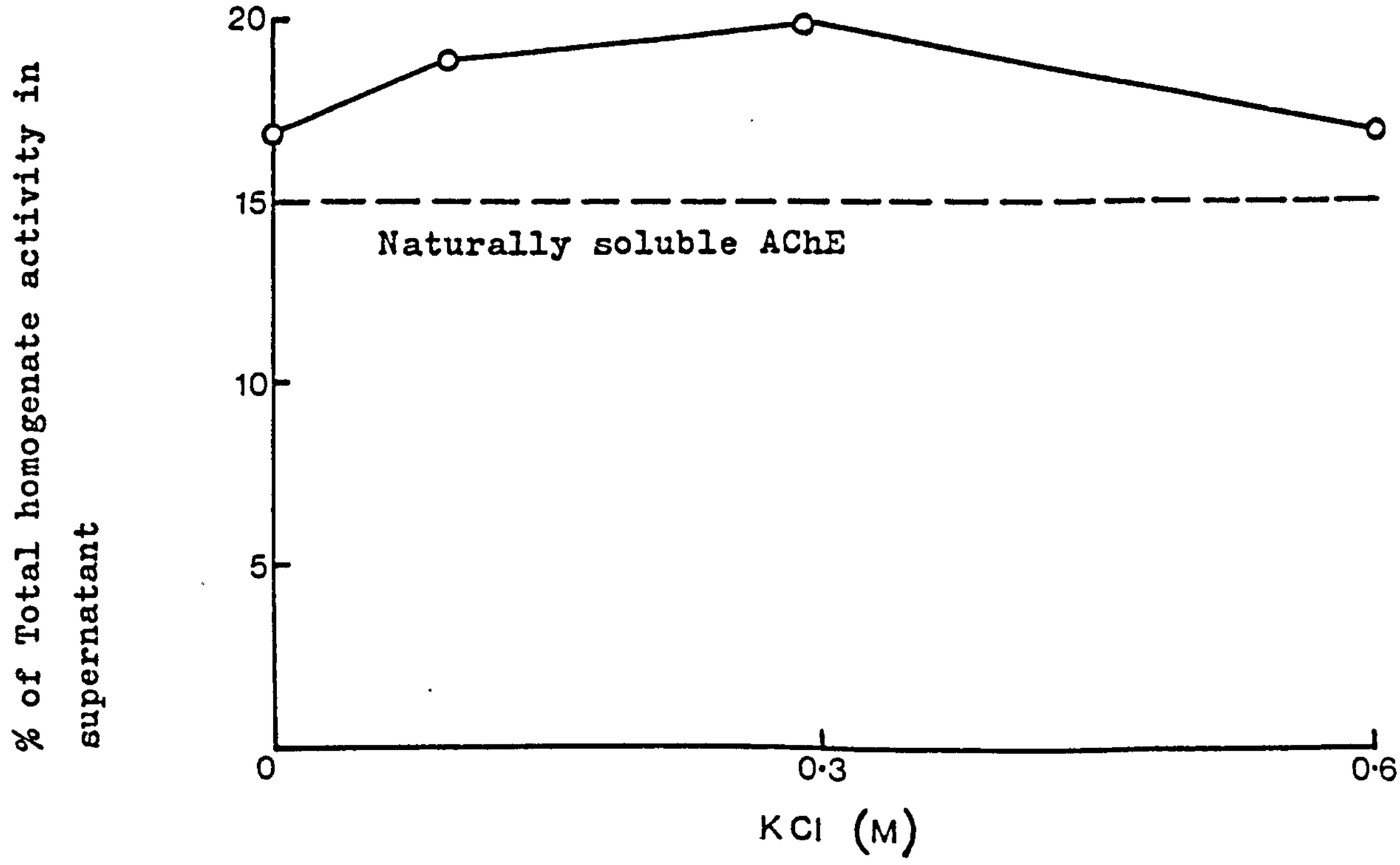


Fig. III.2 Effect of KCl on Triton X-100 (1%, w/v) Solubilization of Acetylcholinesterase



E. Bile Salts

(i) Sodium cholate Sodium cholate was not very effective as a solubilizing agent. When the sodium cholate concentration was increased from 0.5% - 1% (w/v), there was an increase in the yield of the enzyme from 3% to 18.5% of the total homogenate activity. With 1% (w/v) sodium cholate, the amount of enzyme solubilized was greatest, being 18.5% (range, 16.5% - 20.5%) of the total homogenate activity (Fig. III.3).

(ii) Sodium deoxycholate Sodium deoxycholate proved to be the most effective solubilizing agent for membrane-bound AChE present in the white matter of porcine brain.

When the sodium deoxycholate concentration was increased from 0.2% - 0.3% (w/v), the amount of AChE solubilized rose from 69% (range, 66-72%) to 88% (range, 87-89%) of the total homogenate activity. Above 0.3% (w/v) sodium deoxycholate, the AChE activity decreased to 85% (range, 83% - 87%) with 0.4% (w/v) sodium deoxycholate and it further decreased to 76% (range, 74% - 78%) with 0.5% (w/v) sodium deoxycholate (Fig. III.4). These results also show that 100% of the AChE activity was recovered when either 0.3% or 0.4% (w/v) sodium deoxycholate were used in the extraction buffer (summation of the naturally soluble AChE activity and sodium deoxycholate solubilized AChE activity). Another disadvantage of increasing the sodium deoxycholate concentration is the problem of lipid coming out of solution, which makes it difficult to remove the supernatant in which the enzyme is found.

It was found that there was no difference in the enzyme activity between the homogenate with or without the solubilizing agents mentioned. When one of the solubilizing agents such as

Fig. III.3 Effect of Sodium cholate on Solubilization of Acetylcholinesterase

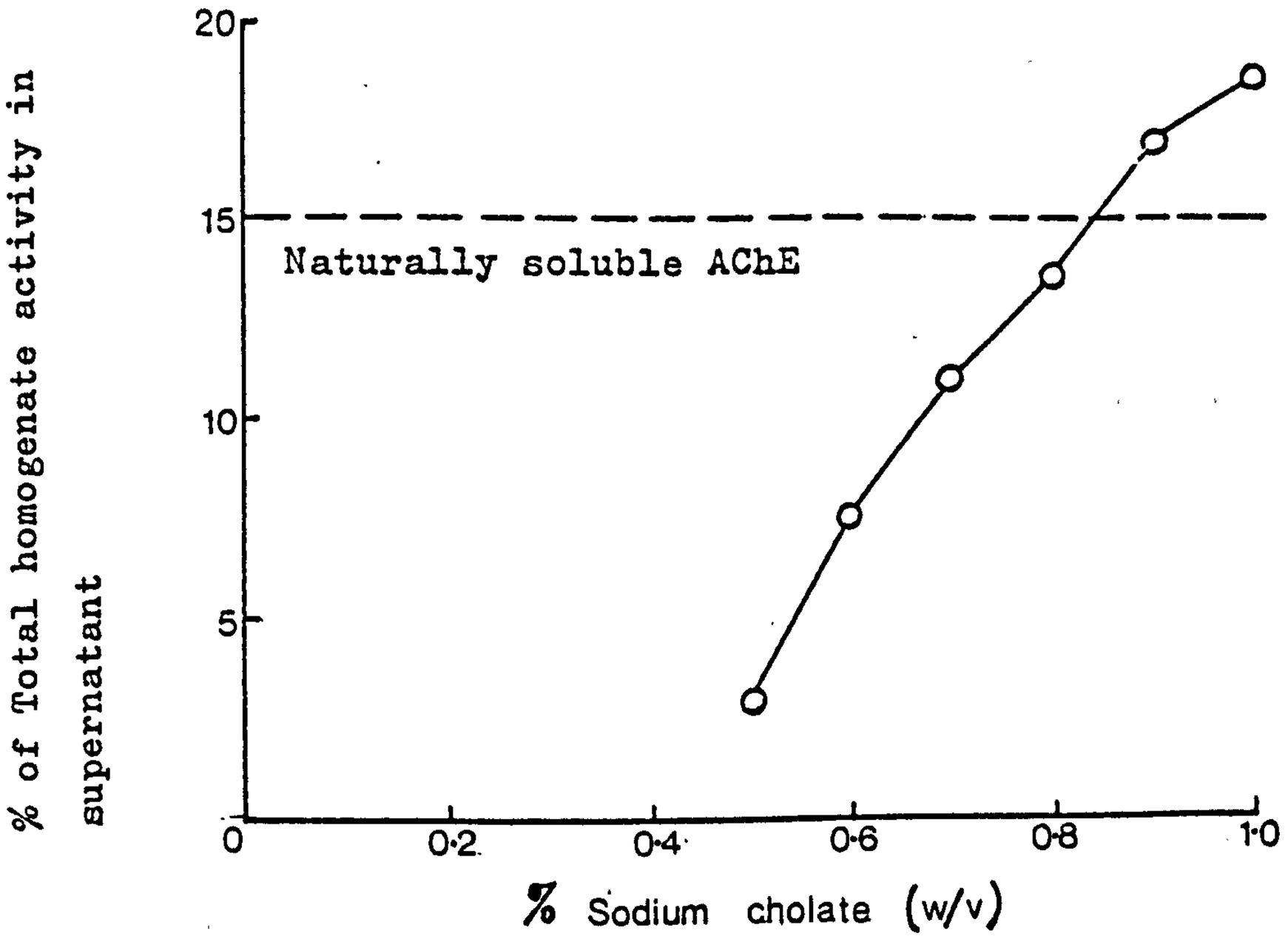
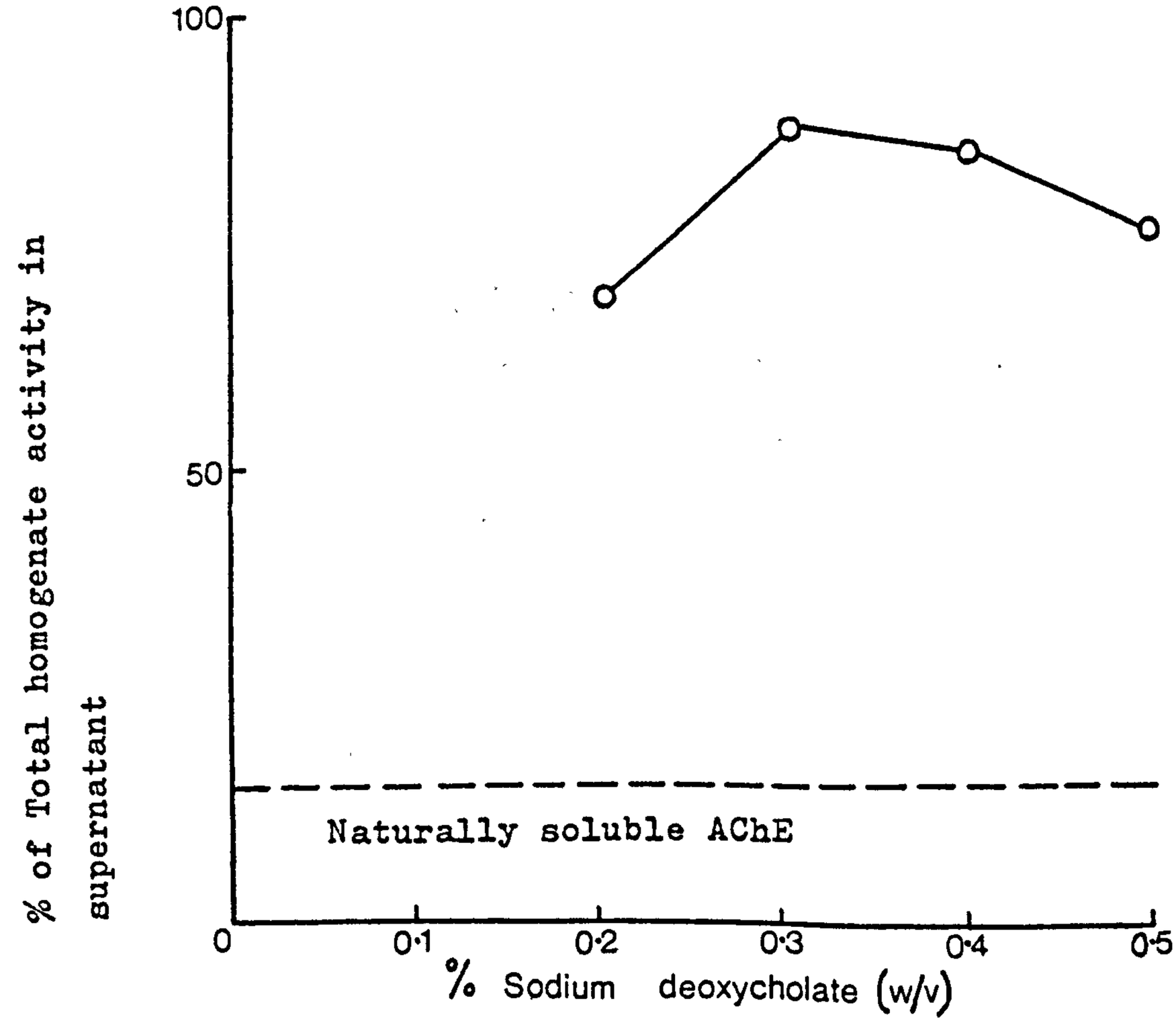


Fig. III.4 Effect of Sodium deoxycholate on Solubilization of Acetylcholinesterase



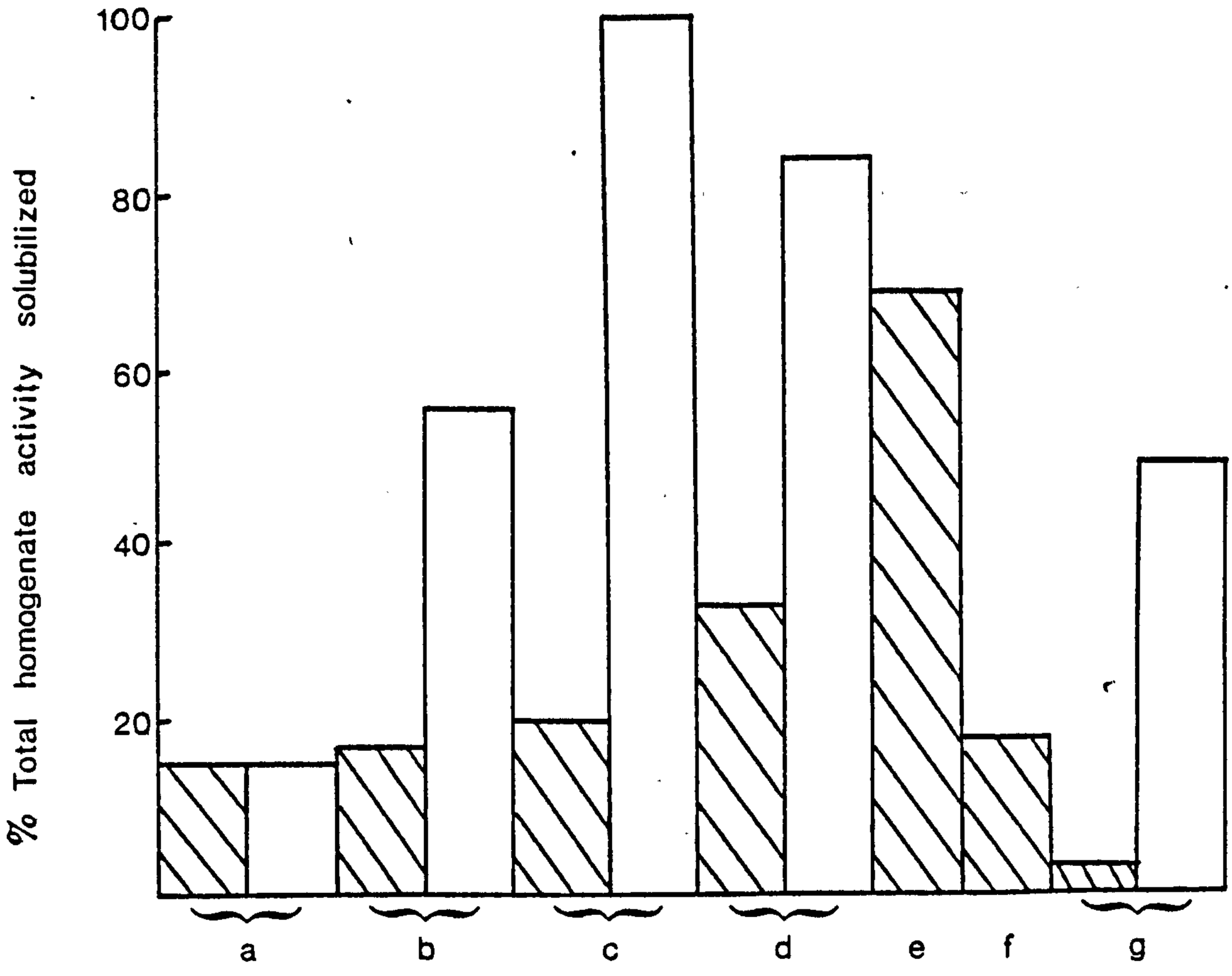
EDTA, Triton X-100, lysolecithin, sodium cholate and sodium deoxycholate was included in the extraction buffer, there was no difference in the homogenate enzyme activity and so no activation or inhibition of the enzyme had occurred. The AChE activity in the 100,000 g. pellet obtained from centrifugation at 100,000 g. for 1 h. was assayed and it was shown that there was no activation or inhibition of the particulate AChE in any of the enzyme preparations (Table III.1).

The standard procedure adopted for solubilizing the membrane-bound AChE in white matter was extraction of the tissue with 0.2% (w/v) sodium deoxycholate and using this method 69% (range, 66% - 72%) of the total homogenate activity was solubilized by the action of the detergent. As mentioned earlier, even though increased concentrations of sodium deoxycholate increased the solubilization of AChE, the problem of lipid coming out of solution made it impractical. The results obtained with the different solubilization procedures are summarized in Fig. III.5 and Table III.2. Figure III.5 also includes data obtained for AChE in porcine grey matter which will be discussed in Section V.

F. Kinetics

(i) Specificity of enzyme preparations The substrate specificity of the sodium deoxycholate solubilized AChE and of the naturally soluble AChE were examined. This was carried out by measuring the rates of hydrolysis of a number of substrates by the enzyme preparations using the pH-stat. From results obtained, both preparations gave fairly similar values (Table III.3). Data obtained for AChE in porcine grey matter (cerebral cortex) was also included in Table III.3 and this data will be discussed in Section V.

Fig. III.5 Solubilization of Brain Acetylcholinesterase



(a) Dilute buffer (Naturally soluble AChE)

(b) Triton X-100 (1%, w/v)

(c) 1% Triton X-100/0.3M or 1M KCl

(d) Lysolecithin (12 mmol/l)

(e) Deoxycholic acid (0.2%, w/v), Na salt

(f) Cholic acid (1%, w/v), Na salt

(g) EDTA (1 mmol/l)

white matter

grey matter

Table III.2

Solubilization of Acetylcholinesterase from White Matter of Porcine Brain

Enzyme preparation	Activity /g. wet wt. u/g. wet wt.	Yield of activity %	Protein mg/g.wet wt.	Yield of protein %	Specific activity u/mg. protein	Number of Experiments
Homogenate (10%, w/v)	3.80 ± 0.14	100	79.2 ± 2.5	100	0.048	20
100,000 g. supernatant (Aqueous extract)	0.58 ± 0.03	13 - 17	19.3 ± 0.8	22 - 26	0.030	20
Treatment of 100,000 g. pellet with:						
EDTA (1 mmol/l)	0.11 ± 0.005	2.8 - 3.2	4.0 ± 0.2	4.8 - 5.2	0.028	5
Triton X-100 (1%, w/v)	0.60 ± 0.03	16 - 18	4.0 ± 0.1	4.9 - 5.1	0.15	5
Triton X-100 + KCl (1%, w/v) (0.3 mol/l)	0.76 ± 0.04	17.5 - 22.5	3.2 ± 0.1	3.84 - 4.16	0.24	5
Sodium cholate (1%, w/v)	0.70 ± 0.03	16 - 20	4.8 ± 0.2	5.8 - 6.2	0.145	5
Lysolecithin (12 mmol/l)	1.30 ± 0.05	30 - 36	10.3 ± 0.5	12.6 - 13.4	0.13	5
Sodium deoxycholate (0.2%,w/v)	2.60 ± 0.13	66 - 72	41.9 ± 1.6	50 - 56	0.062	20

The values given are means ± S.E.M.; u represents μmoles acetylthiocholine iodide hydrolysed per minute.

Table III.3

Hydrolysis of Choline Esters by Acetylcholinesterase in White Matter

Substrate	Rate*		
	White matter AChE		Grey matter AChE
	Naturally soluble	Sodium deoxycholate solubilized	Naturally soluble/ Triton solubilized
Acetylcholine iodide	100.0	100.0	100.0
Acetylthiocholine iodide	111.8	118.6	147.0
Acetyl-β-methylcholine bromide	21.3	22.0	21.7
Propionylcholine iodide	82.4	80.9	83.6
Butyrylcholine iodide	2.0	1.8	0.0
Tributyrin	7.4	8.0	4.6

Average of 4 experiments.

* Rate is expressed as percentage of acetylcholine iodide hydrolysis.

The substrate concentration was 1 mmol/l; the investigations were carried out at 30°C and pH of 7.9 was maintained.

Table III.4

Michaelis constants of Brain (White matter) Acetylcholinesterase

Method of Solubilization	K_m (μM) $\pm$ S.E.M.
Crude homogenate	70 $\pm$ 2.9
Untreated 100,000 g. supernatant	106 $\pm$ 5.6
Sodium deoxycholate solubilization	64 $\pm$ 1.5

The results show the mean value $\pm$ S.E.M. for 4 experiments.

The experiments were carried out at 30°C, pH of 7.9 was maintained and the substrate used was acetylcholine iodide.

(ii) Michaelis constants K_m values (Michaelis constants) were obtained for the crude homogenate, the naturally soluble enzyme and the sodium deoxycholate solubilized enzyme. The Michaelis constants were determined by means of 'Woolf' plots (S/v against S) in conjunction with a regression analysis to determine the best straight line fit, with the aid of a computer. Significant differences were observed in the K_m values of the enzyme extracted by the different methods and the sodium deoxycholate solubilized enzyme possessed the lowest K_m value of 64 μM (Table III.4).

G. Purification of the Enzyme by Affinity Chromatography

Sodium deoxycholate solubilized AChE was used in the MAC-agarose affinity column during the purification of the enzyme by affinity chromatography. This enzyme was used as it was obtained by the most efficient method for solubilization of the membrane-bound AChE.

The affinity column was equilibrated with sodium phosphate buffer (0.03 mol/l, pH 7.0) until all the free ligand had been removed. Then the crude enzyme (100-150 ml.) was applied to the column at a rate of 30 ml/h. The affinity column was washed with sodium phosphate buffer (0.03 mol/l, pH 7.0) until no protein could be detected in the eluate. The AChE inhibitor, decamethonium bromide (10 mmol/l), was then incorporated into the sodium phosphate buffer and the elution of enzyme was continued until no further enzyme activity could be detected in the fractions collected from the column. The eluting medium was then changed to sodium phosphate buffer containing NaCl (1 mol/l) when a further peak of enzyme activity was obtained. The elution profile is shown in Fig. III.6.

The total yield of enzyme from the column amounted to 35%, with 24% of the AChE activity being eluted in the decamethonium peak (peak I) and 11% of the enzyme activity being eluted by 1 mol/l NaCl in the second peak (peak II). The purification of the enzyme in peak I was 300-fold while that in peak II was 200-fold. The enzyme in the first peak was therefore used in subsequent investigation and after dialysis, the peak I enzyme had a specific activity of 18.63 $\mu mol.min^{-1}.mg^{-1}$. A summary of the results obtained is shown in Table III.5.

Fig. III.6 Elution Profile of AChE from MAC-column Using Decamethonium bromide

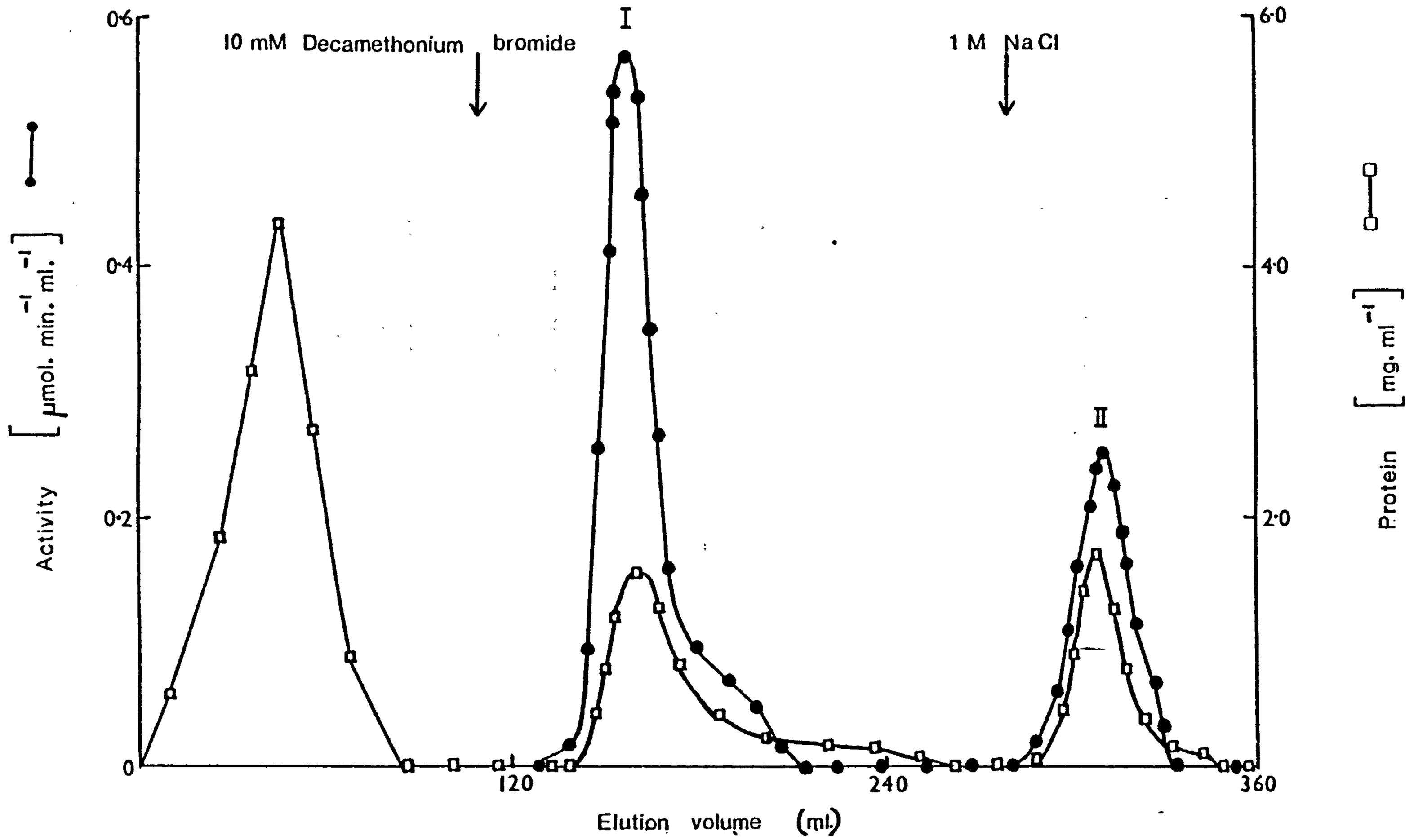


Table III.5

Purification of Acetylcholinesterase on MAC-agarose affinity column

	Protein (mg)	Enzyme activity (u)	Specific activity (u mg^{-1} protein)	Yield (%)	Purification (times)
Total applied	629.0	39.84	0.063	-	-
Recovered in peak I	0.52	9.69	18.63	24.0	300
Recovered in peak II	0.36	4.55	12.64	11.0	200

u represents μmoles acetylthiocholine iodide hydrolysed per minute.

2. ELECTROPHORESIS

A. Starch Block Electrophoresis

The sodium deoxycholate solubilized AChE when electrophoresed on a starch block (without sodium deoxycholate in the block or tank buffer) at pH 8.0 showed one major peak of enzyme activity. (Fig. III.7)

The naturally soluble AChE also showed one major peak of enzyme activity (Fig. III.8). This peak was slightly broader than the peak obtained from electrophoresis of the sodium deoxycholate solubilized enzyme and had a higher negative mobility.

The enzyme purified by affinity chromatography showed exactly the same characteristics as the crude bile salt solubilized enzyme when subjected to electrophoresis on a starch block.

B. Electrophoresis on Rods of Polyacrylamide (7% polyacrylamide gel)

Crude enzyme preparations, peaks of enzyme activity obtained from starch block electrophoresis and purified enzyme were applied to polyacrylamide rods and electrophoresis was carried out. The enzyme band patterns are shown in Fig. III.9.

The sodium deoxycholate solubilized enzyme always showed three characteristic bands of enzyme activity, the fastest moving band being the most densely stained. The peak from starch block electrophoresis of this enzyme when electrophoresed on polyacrylamide rods gave a similar staining pattern to the crude sodium deoxycholate solubilized enzyme.

The staining pattern of the naturally soluble enzyme showed five bands of enzyme activity. The most densely stained zones of

Fig. III.7 Starch Block Electrophoresis of Sodium deoxycholate solubilized Acetylcholinesterase

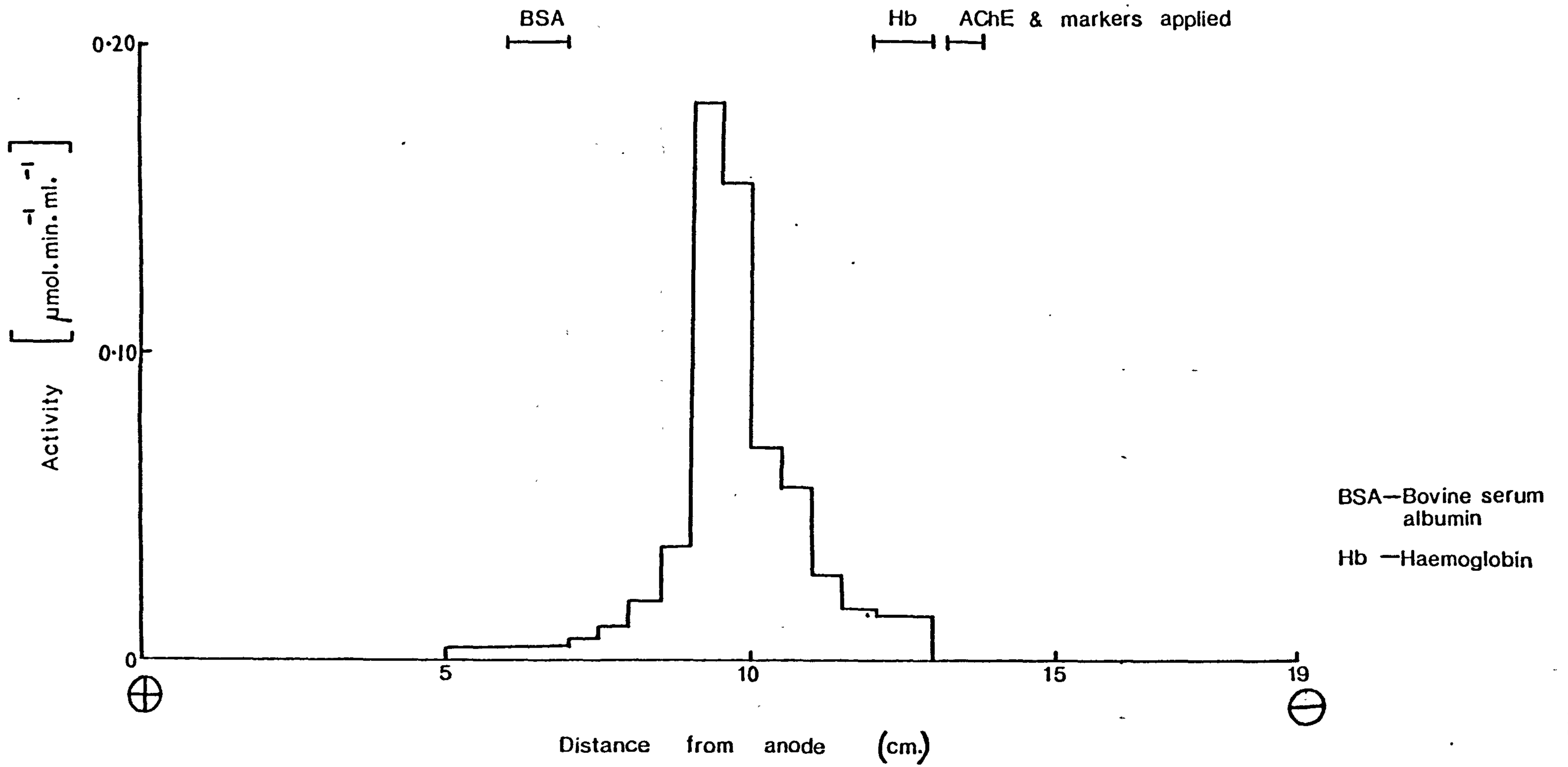
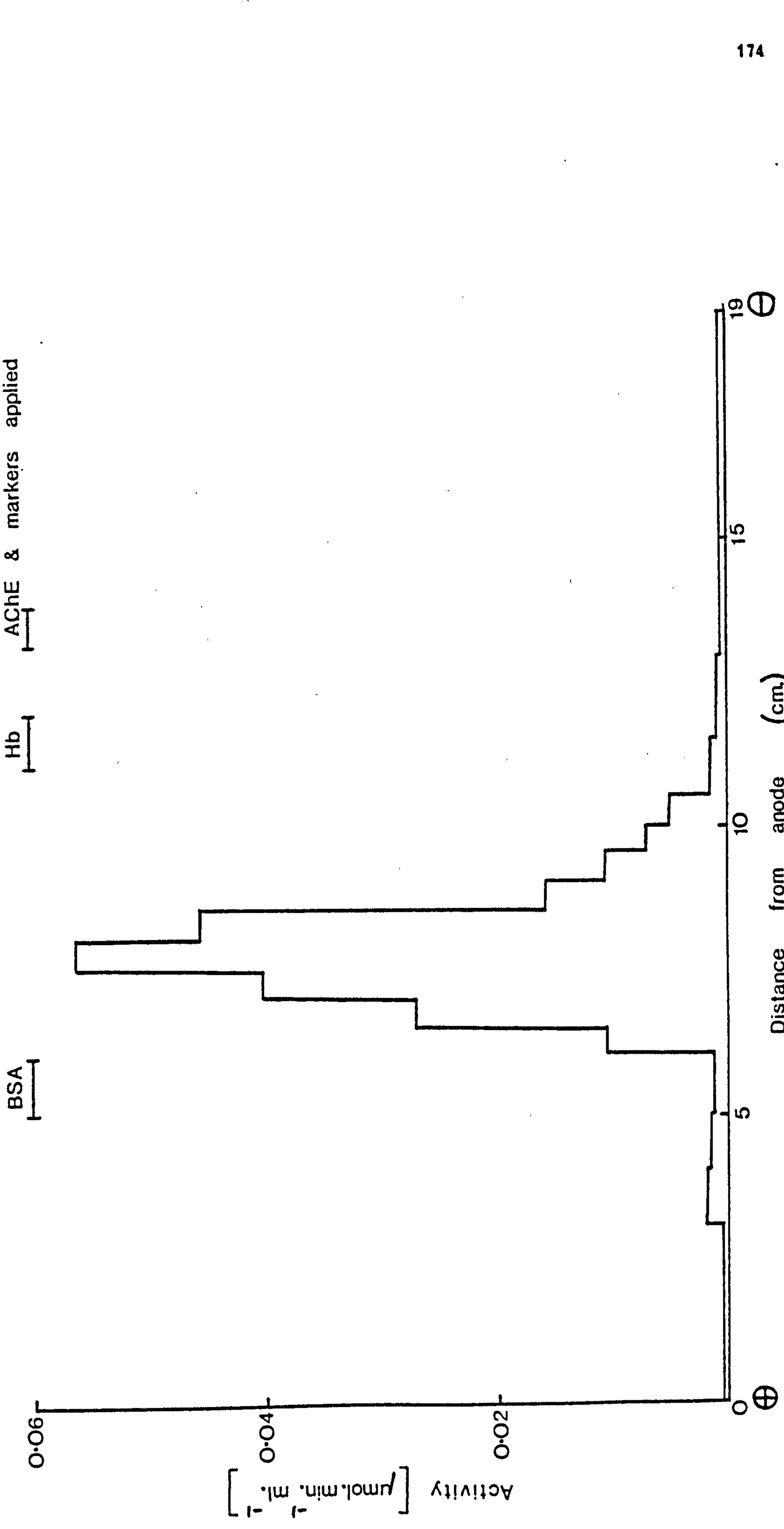


Fig. III.8 Starch Block Electrophoresis of Naturally soluble Acetylcholinesterase



activity corresponded to the densely staining bands of enzyme in the rods containing the sodium deoxycholate solubilized AChE. The peak from starch block electrophoresis of the naturally soluble AChE showed a similar staining pattern to the crude naturally soluble enzyme when it was electrophoresed on polyacrylamide rods.

The affinity chromatography-purified AChE gave two bands of enzyme activity which corresponded to the fastest and slowest moving bands in the staining pattern of both the sodium deoxycholate solubilized and naturally soluble enzyme. However, these bands were sharper and more intensely stained than bands obtained for the other enzyme preparations.

C. Electrophoresis on a Gradient of Polyacrylamide (4% - 24% polyacrylamide gel)

Electrophoresis in gradient polyacrylamide slabs showed multiple bands of enzyme activity in the enzyme preparations. (Fig. III. 10).

On the assumption that the AChE molecules are spherical, the molecular weights obtained for the naturally soluble enzyme were 661,000; 422,000; 266,000 and 120,000. The sodium deoxycholate solubilized enzyme had three bands of activity in common with the naturally soluble AChE with molecular weights at 422,000; 266,000 and 120,000. In addition to these bands, it also had a species with a molecular weight at 181,000. The peaks of activity from the starch block electrophoresis of the sodium deoxycholate solubilized and naturally soluble AChE gave the same staining patterns as enzyme before electrophoresis on starch blocks.

The staining pattern for the electrophoresis of the affinity

chromatography purified enzyme was quite different from the other staining patterns. The bands of enzyme activity were located at distances across the polyacrylamide slab corresponding to molecular weights at 260,000; 181,000; 162,000; 134,000 and 120,000. It was also observed that these bands were sharper and more intensely stained than in other enzyme preparations which were electrophoresed.

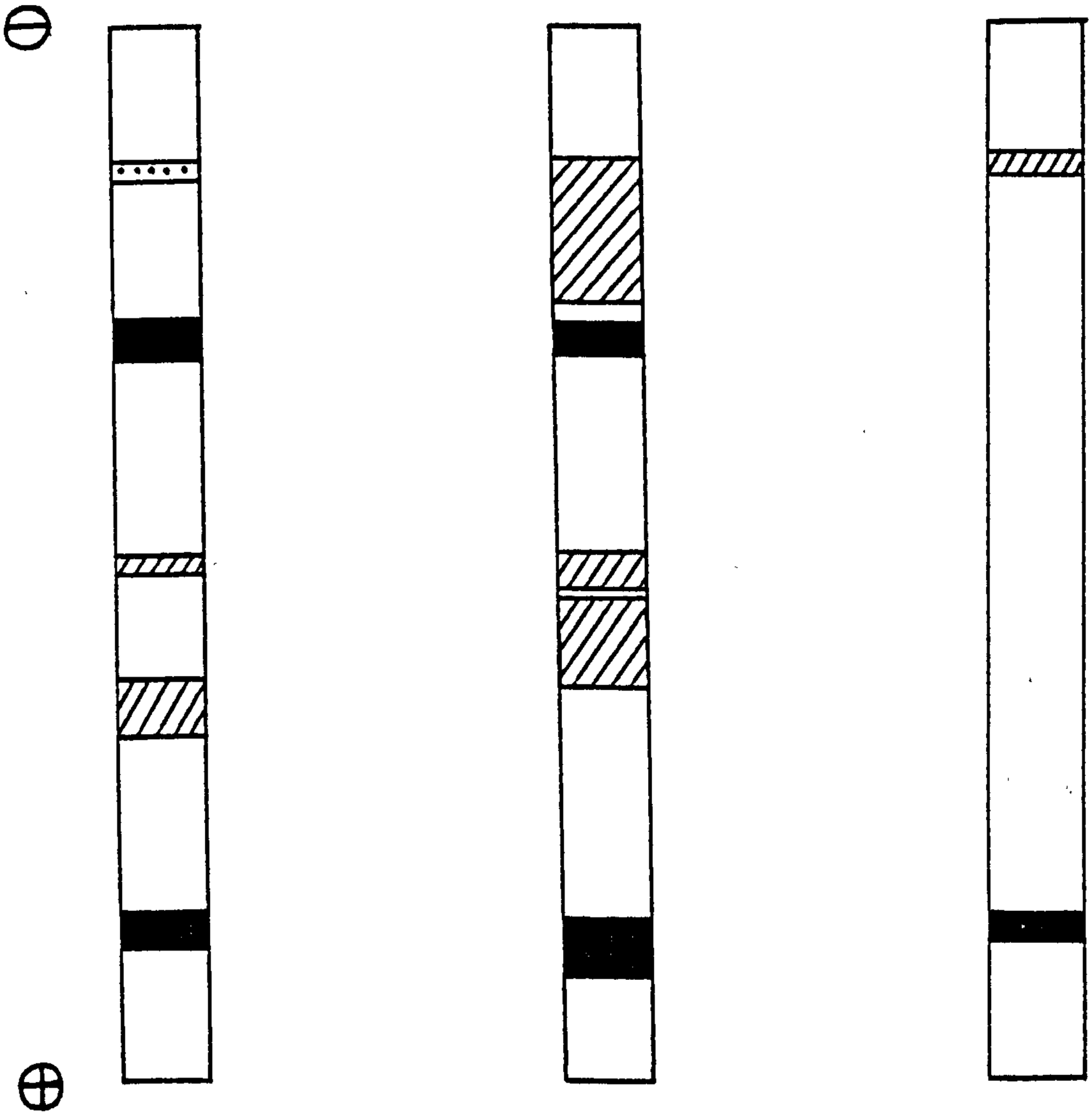
Staining for protein in polyacrylamide rods and slabs showed multiple bands for each enzyme preparation. The staining revealed that the protein present corresponded to most but not all of the AChE bands.

When 10 µmol/l physostigmine sulphate (eserine) was incorporated into the staining system, no AChE activity was detected in the polyacrylamide rods and slabs. This indicates that the activity previously detected when eserine was not included in the staining system was due to a cholinesterase. The use of butyrylthiocholine iodide in place of acetylthiocholine iodide as substrate in the stain showed a few faint bands of activity which did not correspond to those bands produced when acetylthiocholine iodide was used as a substrate in the stain. This observation shows that the majority of the enzyme activity present in the preparations was acetylcholinesterase and not butyrylcholinesterase.

Finally, the incorporation of 30 µmol/l ethopropazine into the staining system had no effect on the bands produced when acetylthiocholine iodide was used as a substrate but it totally inhibited the bands observed when butyrylthiocholine iodide was used as a substrate. This indicates that the enzyme detected was acetylcholinesterase and not butyrylcholinesterase.

Fig. III.9 Polyacrylamide Gel Rod Electrophoresis of Acetylcholinesterase

Electrophoresis was carried out at 2.5 mA per tube for 1½ h.



Staining for AChE

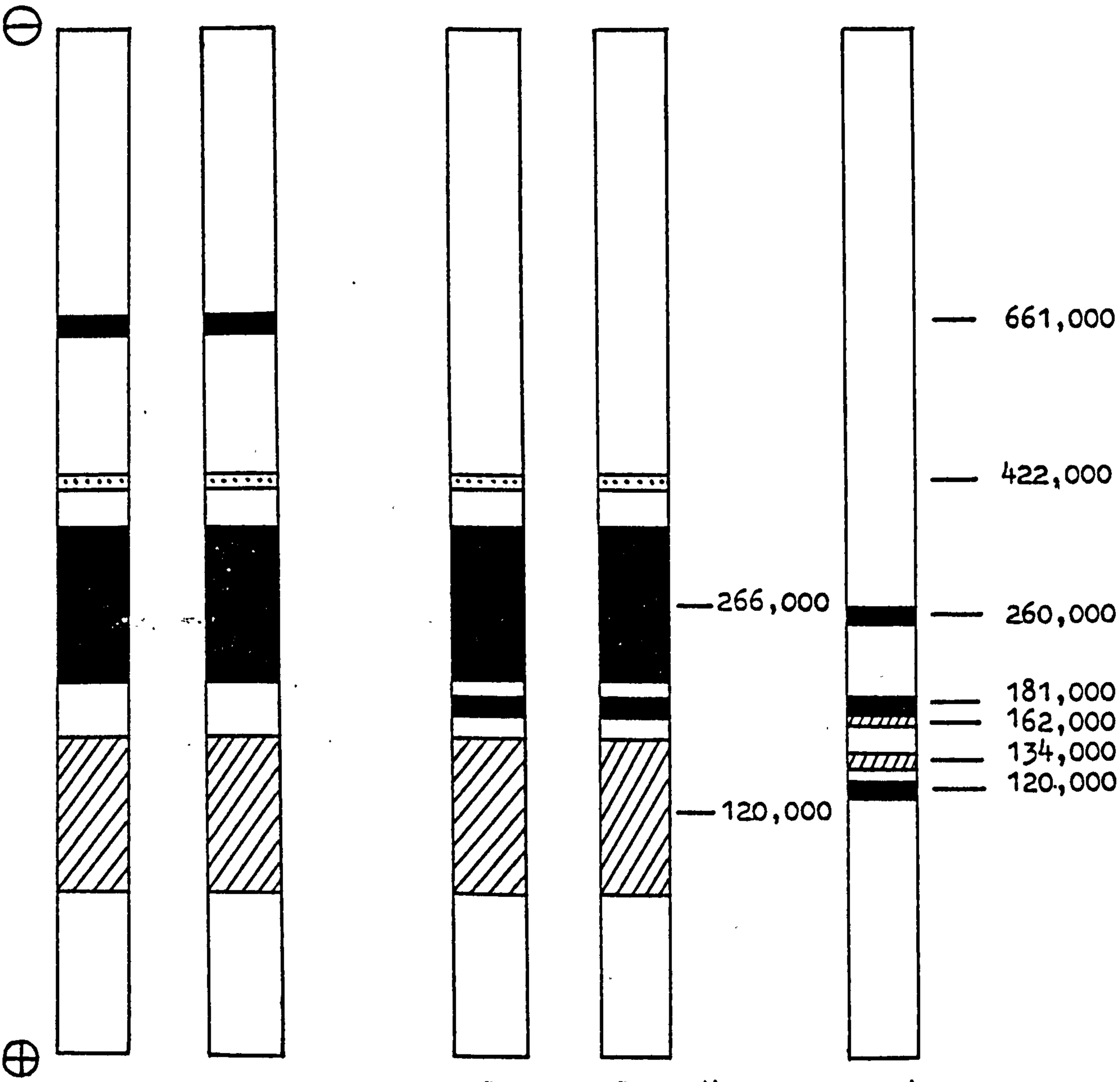
Light staining

Intermediate staining

Dense staining

Fig. III.10 Polyacrylamide Gradient Slab Gel Electrophoresis of Acetylcholinesterase

Electrophoresis was carried out at 100 v. for 24 h.



Naturally soluble

Naturally soluble peak from starch block

Sodium deoxycholate solubilized

Sodium deoxycholate solubilized peak from starch block

Sodium deoxycholate solubilized, purified by affinity chromatography

Staining for AChE

Light staining

Intermediate staining

Dense staining

3. SUCROSE DENSITY GRADIENT CENTRIFUGATION

Three standard proteins, muramidase, alcohol dehydrogenase and catalase were run on sucrose density gradients and a direct relationship was observed between their sedimentation coefficient and the distance migrated down the centrifuge tube. (Fig. III.11). Using this relationship, the sedimentation coefficients of the enzyme preparations were calculated by comparing the migration distances of the marker protein catalase, and AChE.

When the sodium deoxycholate solubilized AChE was centrifuged on sucrose gradients, it migrated to the bottom of the gradient suggesting the presence of a high molecular weight species. However, when sodium deoxycholate (0.2%,w/v) was incorporated into the sucrose, one peak of enzyme activity was resolved corresponding to 10.6 S or a species of molecular weight 215,000 if the enzyme is spherical in shape. (Fig. III.12).

The naturally soluble AChE when centrifuged on sucrose gradients showed one peak of enzyme activity at 10.4 S which corresponded to a species of molecular weight 209,000. (Fig. III.13).

The peaks of activity of sodium deoxycholate solubilized enzyme and naturally soluble enzyme obtained from starch block electrophoresis were also subjected to sucrose density gradient centrifugation. In both cases, the peaks from the electrophoresis gave a sedimentation coefficient value of 11 S which corresponded to a molecular species of molecular weight 227,000.

The affinity chromatography-purified AChE was also centrifuged on a sucrose gradient and it was found to have the same sedimentation coefficient value as the crude sodium deoxycholate solubilized enzyme, before and after starch block electrophoresis.

Fig. III.11 Sedimentation Behaviour of Muramidase, Alcohol dehydrogenase and Catalase on 5 ml. Sucrose Density Gradients

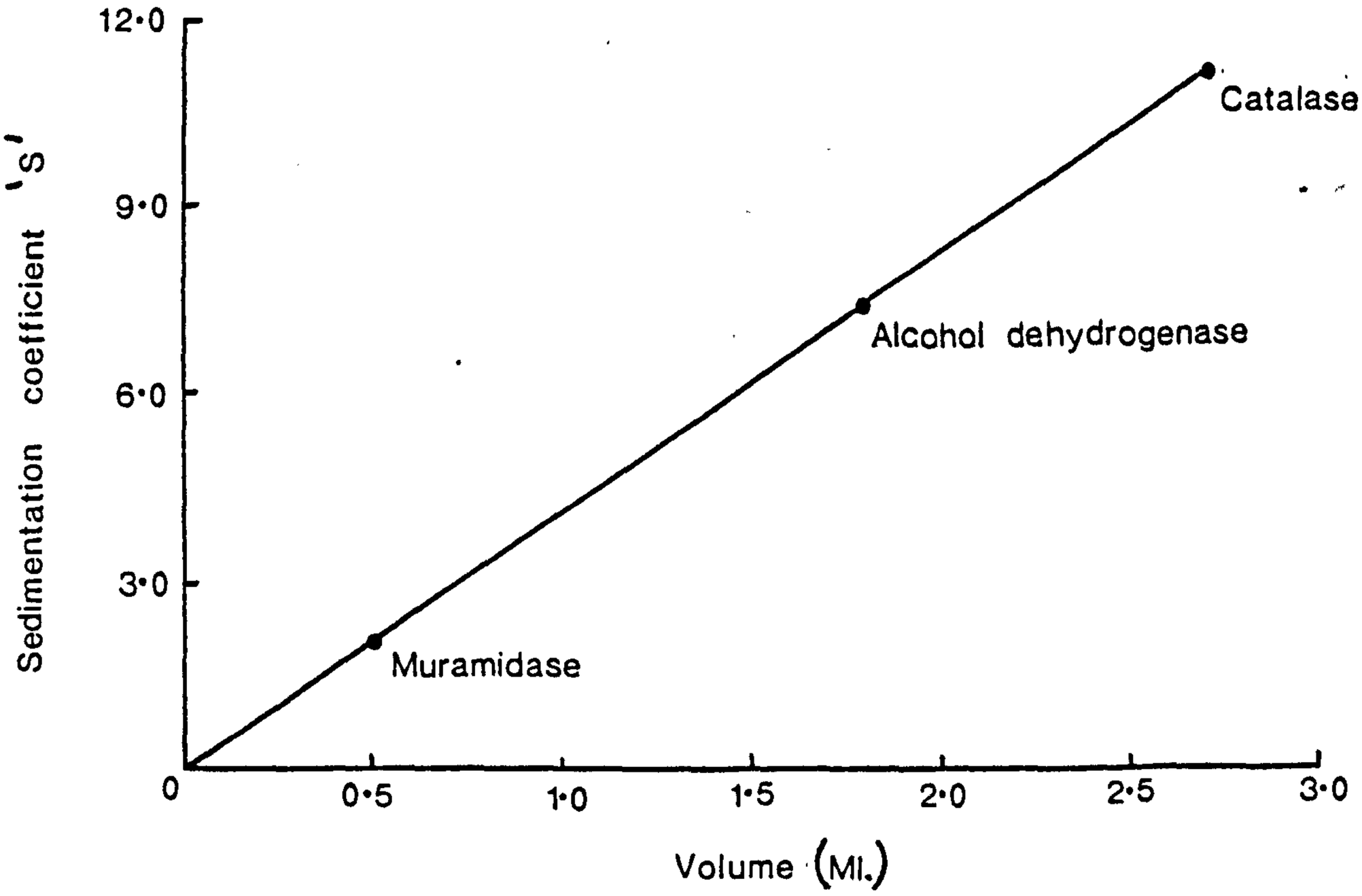


Fig. III.12 Sucrose Density Gradient Centrifugation of Sodium deoxycholate solubilized Acetylcholinesterase

(Δ Abs.= Change in absorbance $_{412\ nm}$)

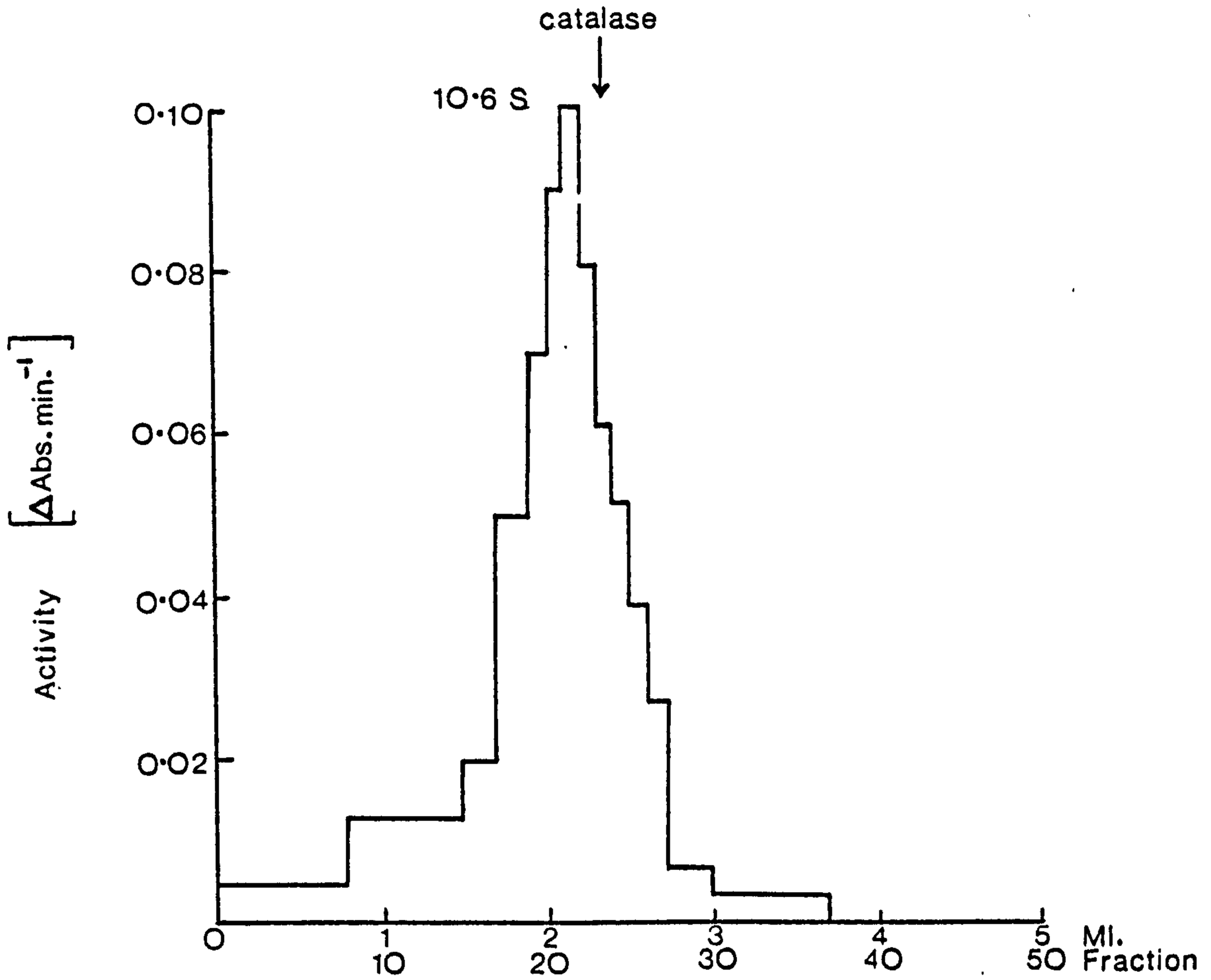
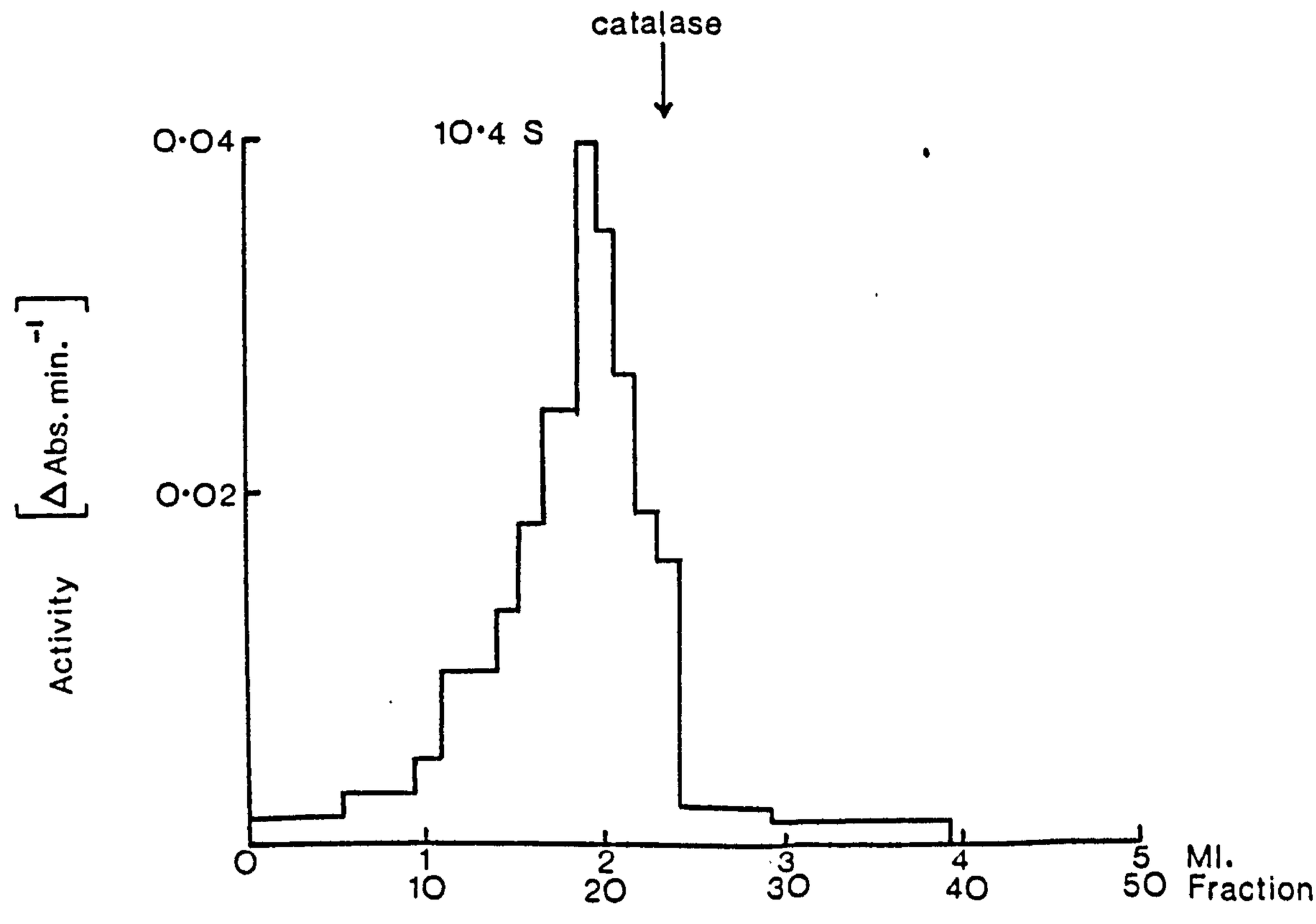


Fig. III.13 Sucrose Density Gradient Centrifugation of Naturally soluble Acetylcholinesterase



SECTION IV

RESULTS

ACETYLCHOLINESTERASE FROM RAT MUSCLE

1. SOLUBILIZATION OF ACETYLCHOLINESTERASE

A. Aqueous Media

Extraction of a 20% (w/v) homogenate of rat gastrocnemius muscle with water or 0.03 mol/l sodium phosphate buffer (pH 7.0) solubilized 51% (range, 49% - 53%) of the total homogenate activity. This fraction was referred to as the 'naturally soluble' enzyme. Further extraction of the pellet obtained from the 100,000 g. centrifugation, with the same medium failed to solubilize any more of the enzyme.

The remaining AChE activity in the 100,000 g. pellet was also determined. The pellet was resuspended in sodium phosphate buffer (0.03 mol/l; pH 7.0) and its enzyme activity assayed. It was found that 49% (range, 48% - 50%) of the total homogenate activity was present in the pellet and this enzyme activity was assumed to be membrane-bound (Table IV.1).

B. Triton X-100

An attempt was then made to bring the membrane-bound enzyme into solution with the detergent Triton X-100. Extraction of the AChE activity from the 100,000 g. pellet with sodium phosphate buffer (0.03 mol/l, pH 7.0) containing 1% (w/v) Triton X-100 solubilized 75% (range, 73% - 77%) of the total homogenate activity. As Triton X-100 was added in increasing concentration (0.1% - 2%, w/v), the amount of enzyme solubilized increased from 44% to 76% of the total homogenate activity (Fig. IV.1). The total AChE activity obtained by extracting the muscle with 1% (w/v) Triton X-100 was therefore 126% of the total homogenate activity,

of which 51% was naturally soluble AChE and 75% was AChE activity which was solubilized by the detergent. These results would suggest that some activation of the enzyme has occurred and indeed a 15% - 25% activation of the AChE activity was observed when the enzyme activity of the uncentrifuged homogenate was assayed in the presence of 1% (w/v) Triton X-100.

The standard procedure adopted for solubilization of the membrane-bound AChE in rat muscle was therefore extraction with 1% (w/v) Triton X-100, and using this procedure, 75% (range, 73% - 77%) of the total homogenate activity was solubilized, which represented a significant activation of the enzyme (Table IV.2).

Table IV.1

Acetylcholinesterase in the Naturally Soluble Fraction

Fraction	Activity/g. wet wt. u/g. wet wt.	Yield of activity %	Protein mg/g. wet wt.	Yield of protein %	Specific activity u/mg. protein
Homogenate (20%, w/v)	0.29 ± 0.01	100	134 ± 6	100	0.0021
100,000 g. pellet	0.135 ± 0.01	48 - 50	78 ± 4	55 - 61	0.0017
100,000 g. supernatant (naturally soluble AChE)	0.14 ± 0.006	49 - 53	50 ± 2.5	35.5 - 38.5	0.0028

The results show the mean value ± S.E.M. for 10 experiments.

u represents μmoles acetylthiocholine iodide hydrolysed per minute.

Fig. IV.1 Effect of Triton X-100 on Solubilization of Acetylcholinesterase

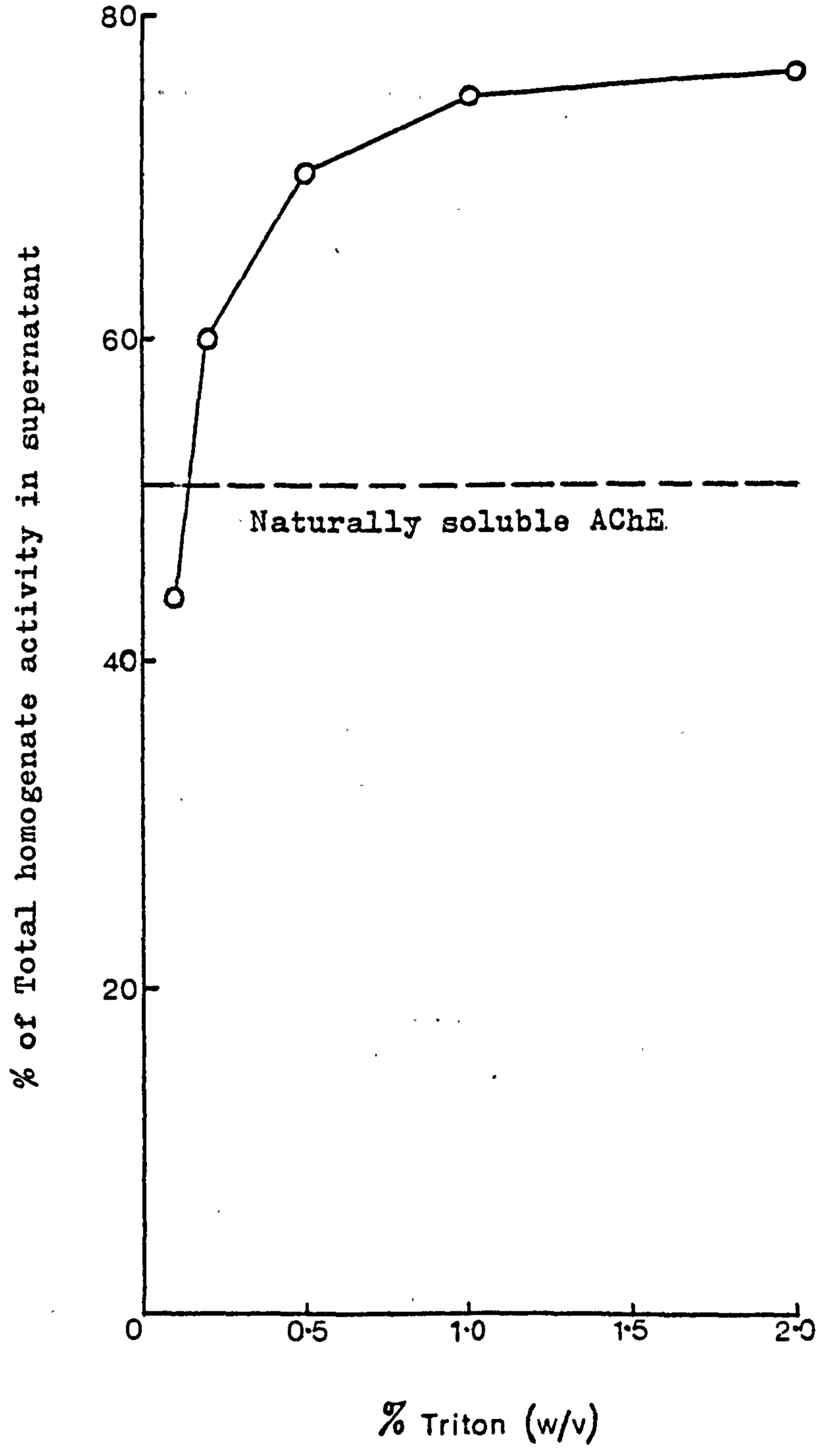


Table IV.2

Acetylcholinesterase in the Triton solubilized Fraction

Fraction	Activity/g. wet wt. u/g. wet wt.	Yield of activity %	Protein mg/g. wet wt.	Yield of protein %	Specific activity u/mg. protein
Homogenate (20%, w/v)	0.29 ± 0.01	100	134 ± 6	100	0.0021
100,000 g. pellet	0.135 ± 0.01	48 - 50	78 ± 4	55-61	0.0017
100,000 g. supernatant from Triton X-100 treatment of resuspended pellet	0.21 ± 0.009	73 - 77	16 ± 0.6	11.3-12.7	0.013

The results show the mean value ± S.E.M. for 10 experiments.

u represents μmoles acetylthiocholine iodide hydrolysed per minute.

2. KINETIC PROPERTIES

A. Substrate Specificity of the Enzyme Preparations

The specificity of the detergent solubilized AChE and of the naturally soluble AChE for a number of substrates was investigated. This was accomplished by measuring the rates of hydrolysis of each substrate at 1 mmol/l by the enzyme preparations using a pH-stat. The results obtained show that both preparations gave fairly similar values. (Table IV.3)

B. Michaelis Constants

The Michaelis constants (K_m values) of the enzyme preparations were determined using the pH-stat assay, and at low substrate concentrations a second burette was utilized as described in the 'methods' section. The K_m values were calculated from 'Woolf' plots (S/v against S) in conjunction with a regression analysis to determine the best straight line fit, using a computer.

The K_m values for the enzyme preparations are shown in Table IV.4. Some differences were observed in the K_m values and the Triton solubilized AChE was shown to have the lowest K_m value (72 μM).

C. Arrhenius Plots of Acetylcholinesterase Preparations

Arrhenius plots of the acetylcholinesterase preparations between the temperatures of 10°C - 40°C were determined using the pH-stat. The V_{max} was obtained from S/v versus S plots (Woolf plots), calculated by a computer, and Log V_{max} was then plotted against the reciprocal of the absolute temperature.

The membrane preparation was obtained by resuspending the 100,000 g. pellet from the centrifugation of a 20% (w/v) muscle homogenate in 0.03 mol/l sodium phosphate buffer, pH 7.0. From the Arrhenius plot of the membrane AChE, two straight lines with a pronounced break at 26°C (transition temperature) were obtained. (Fig. IV.2). The activation energies of this enzyme were calculated to be 21 kJ mol^{-1} and 35 kJ mol^{-1}, from 25°C - 40°C and from 10°C - 25°C respectively. (Table IV.5)

The Triton solubilized AChE was found to have an Arrhenius plot in which the break was abolished and the activation energy was 24 kJ mol^{-1} over the temperature range 10°C - 40°C. (Fig. IV.3)

Similarly, the naturally soluble enzyme demonstrated no break in its Arrhenius plot and the energy of activation was found to be 9.6 kJ mol^{-1} over the temperature range 10°C - 40°C. (Fig. IV.4).

Table IV.3

Hydrolysis of Various Choline Esters by Muscle Acetylcholinesterase

Substrate	Rate*	
	Naturally soluble	Triton solubilized
Acetylcholine iodide	100	100
Acetylthiocholine iodide	145.0	133.5
Acetyl-β-methylcholine bromide	21.0	20.7
Propionylcholine iodide	90.0	84.1
Butyrylcholine iodide	3.0	2.5
Tributyrin	15.0	13.8

Average of 4 experiments.

* Rate is expressed as percentage of acetylcholine iodide hydrolysis.

The substrate concentration was 1 mmol/l; the experiments were carried out at 30°C and pH of 7.6 was maintained.

Table IV.4

Michaelis constants of Muscle Acetylcholinesterase

Method of Solubilization	K_m (μM) $\pm$ S.E.M.
Crude homogenate	84 $\pm$ 1.7
Untreated 100,000 g. supernatant	110 $\pm$ 5.2
Triton solubilized	72 $\pm$ 1.3

The results show the mean value $\pm$ S.E.M. for 4 experiments.

The experiments were carried out at 30°C, pH of 7.6 was maintained and the substrate used was acetylcholine iodide.

Fig. IV.2 Arrhenius Plot of Membrane Acetylcholinesterase

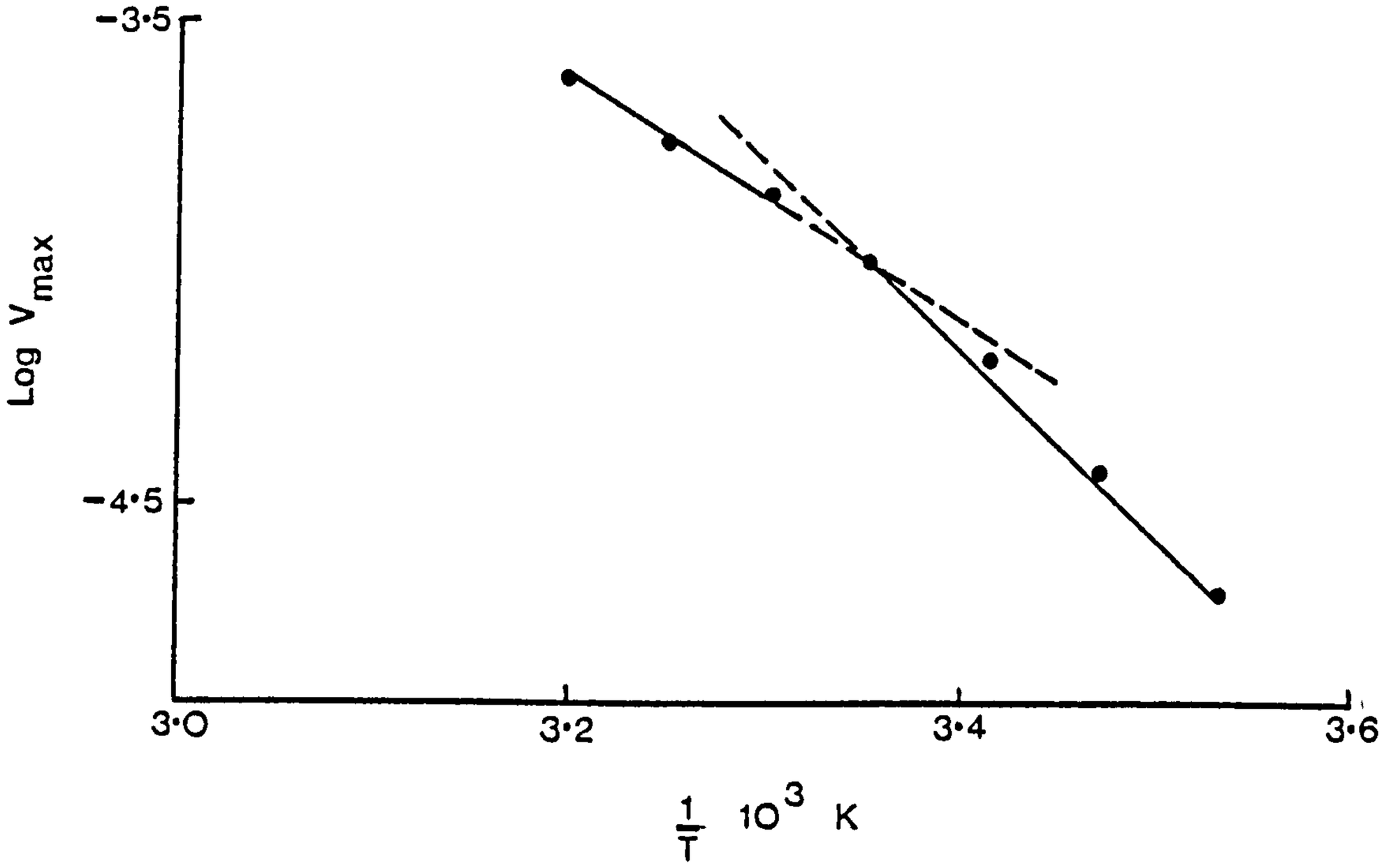


Fig. IV.3 Arrhenius Plot of Triton solubilized Acetylcholinesterase

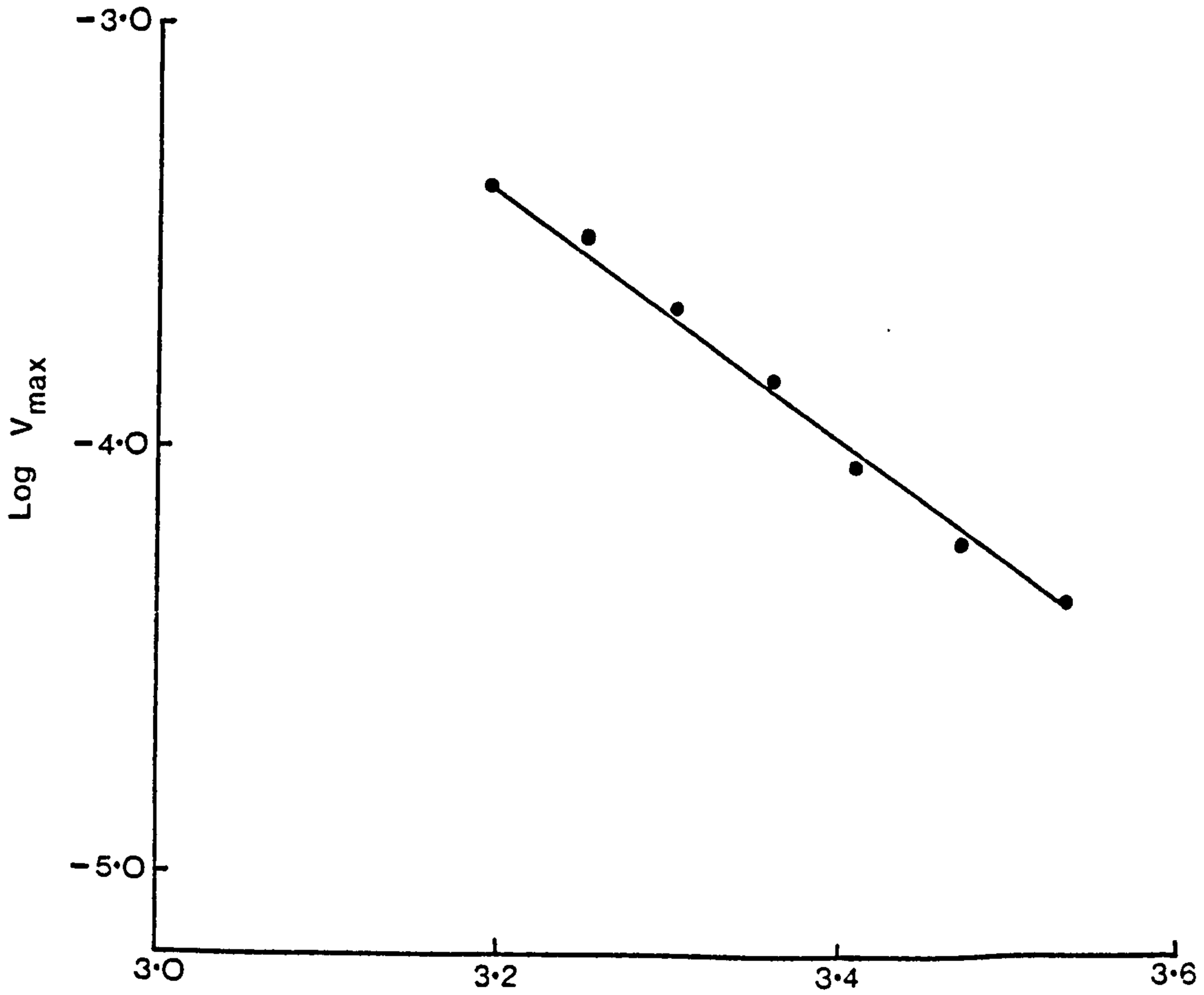


Fig. IV.4 Arrhenius Plot of Naturally soluble Acetylcholinesterase

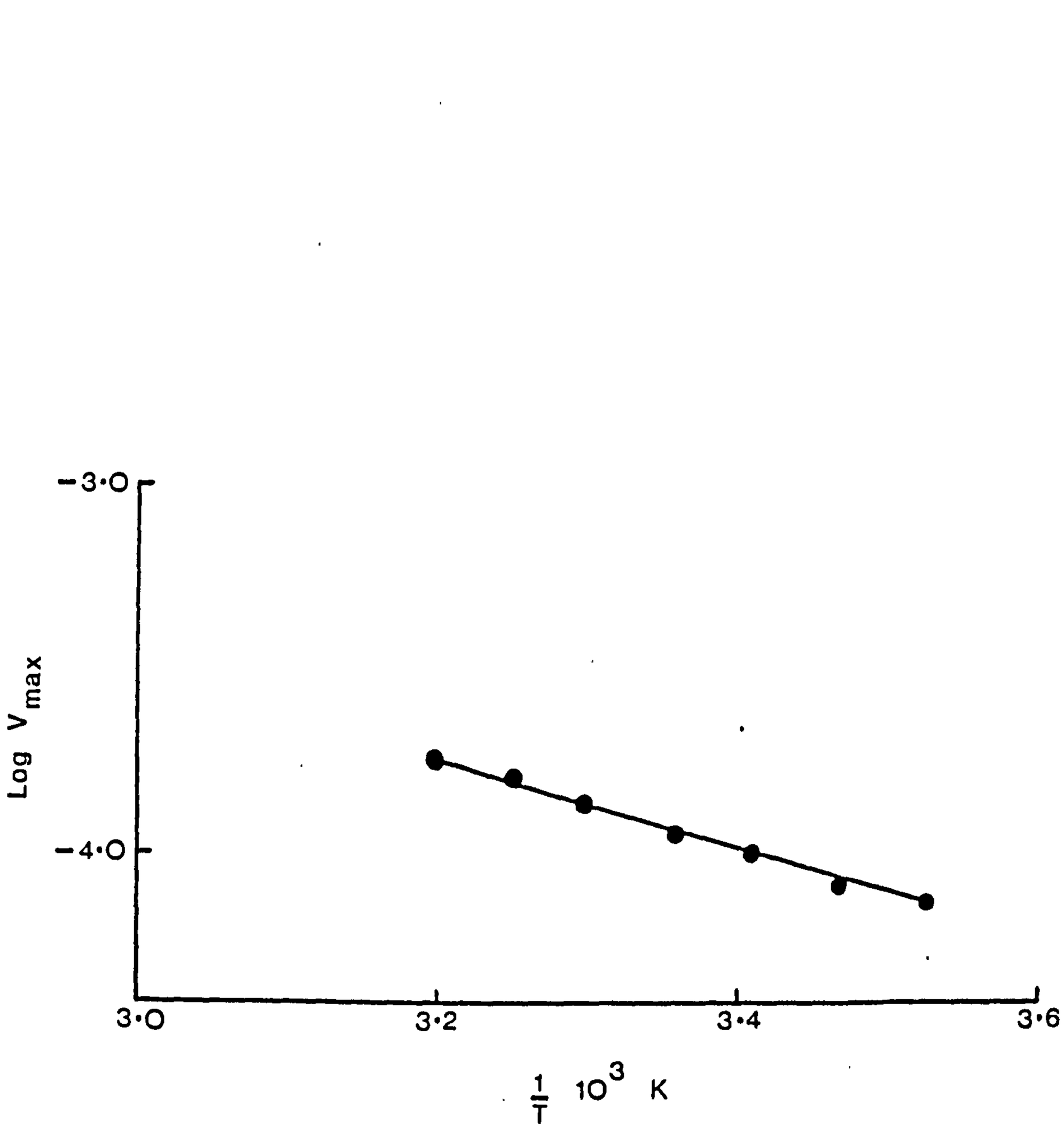


Table IV.5

Activation energies of membrane and soluble muscle acetylcholinesterase

Enzyme preparation	Activation energy (kJ mol^{-1})
Membrane suspension	21 & 35
Aqueous extract	9.6
Triton extract	24

3. ELECTROPHORESIS

A. Starch Block Electrophoresis

When the Triton solubilized enzyme was electrophoresed on a starch block with the detergent excluded from the system, one peak of enzyme activity with a low negative mobility was obtained near the origin. By incorporating Triton X-100 into the starch block and tank buffer, the enzyme was resolved into two entities; a species located where the enzyme was applied (Peak II) and a species with a high negative mobility (Peak I). Fig. IV.5 shows the elution profile of this enzyme preparation.

The naturally soluble AChE was also resolved into two separate peaks of enzyme activity with similar mobilities to the peaks obtained with the Triton solubilized enzyme (Fig. IV.6). However, in the case of the naturally soluble form of the enzyme, detergent was excluded from the starch block and tank buffer.

B. Polyacrylamide Gel Electrophoresis

Electrophoresis in polyacrylamide gradient slabs (4% - 24% polyacrylamide gel) showed a large number of enzymically active species covering a wide range of molecular weights (Fig. IV.7).

The Triton solubilized AChE had seven bands of activity in common with the naturally soluble enzyme, with molecular weights at 733,000; 521,000; 394,000; 355,000; 316,000; 282,000 and 108,000, assuming that the enzyme molecules are spherical. In addition to these forms, the detergent solubilized enzyme had two other species with molecular weights at 225,000 and 180,000.

The naturally soluble enzyme had four other bands of enzyme

activity in addition to the ones mentioned corresponding to molecular weights of 174,000; 124,000; 102,000 and 97,000.

Peak II from the starch block electrophoresis of the Triton solubilized or naturally soluble AChE showed a species of very high molecular weight which just entered the gel. Peak I from the starch block electrophoresis of the enzyme preparations gave the same elution pattern as enzyme before electrophoresis on a starch block, with only a few minor differences in the relative staining intensity of some of the bands.

When physostigmine sulphate (10 µmol/l) or ethopropazine (30 µmol/l) were incorporated into the staining system, their effects on the enzyme proved that the enzyme was a cholinesterase and also proved further that it was acetylcholinesterase.

Fig. IV.5 Starch Block Electrophoresis of Triton solubilized Acetylcholinesterase
(1% (w/v) detergent included in block)

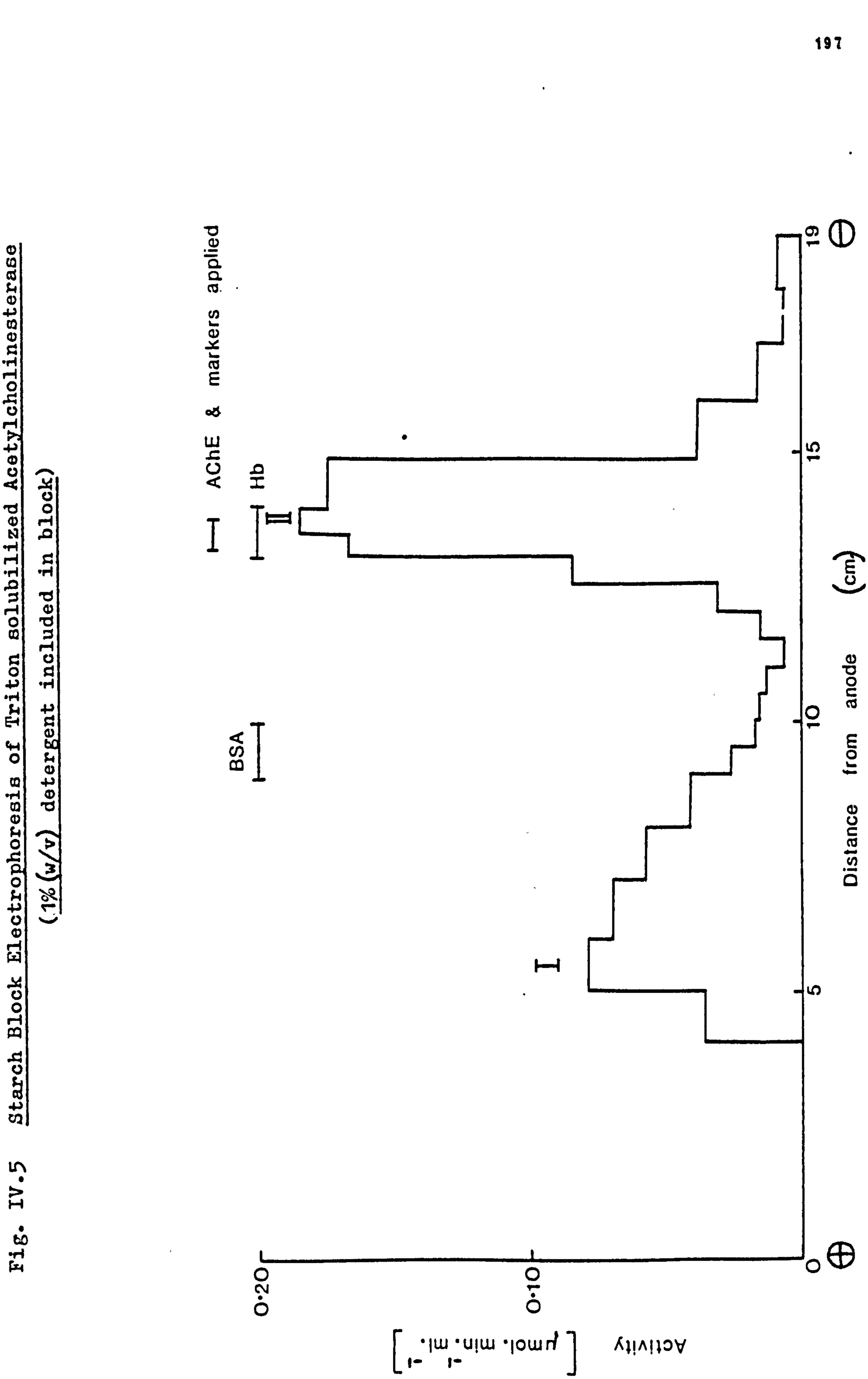


Fig. IV.6 Starch Block Electrophoresis of Naturally soluble Acetylcholinesterase

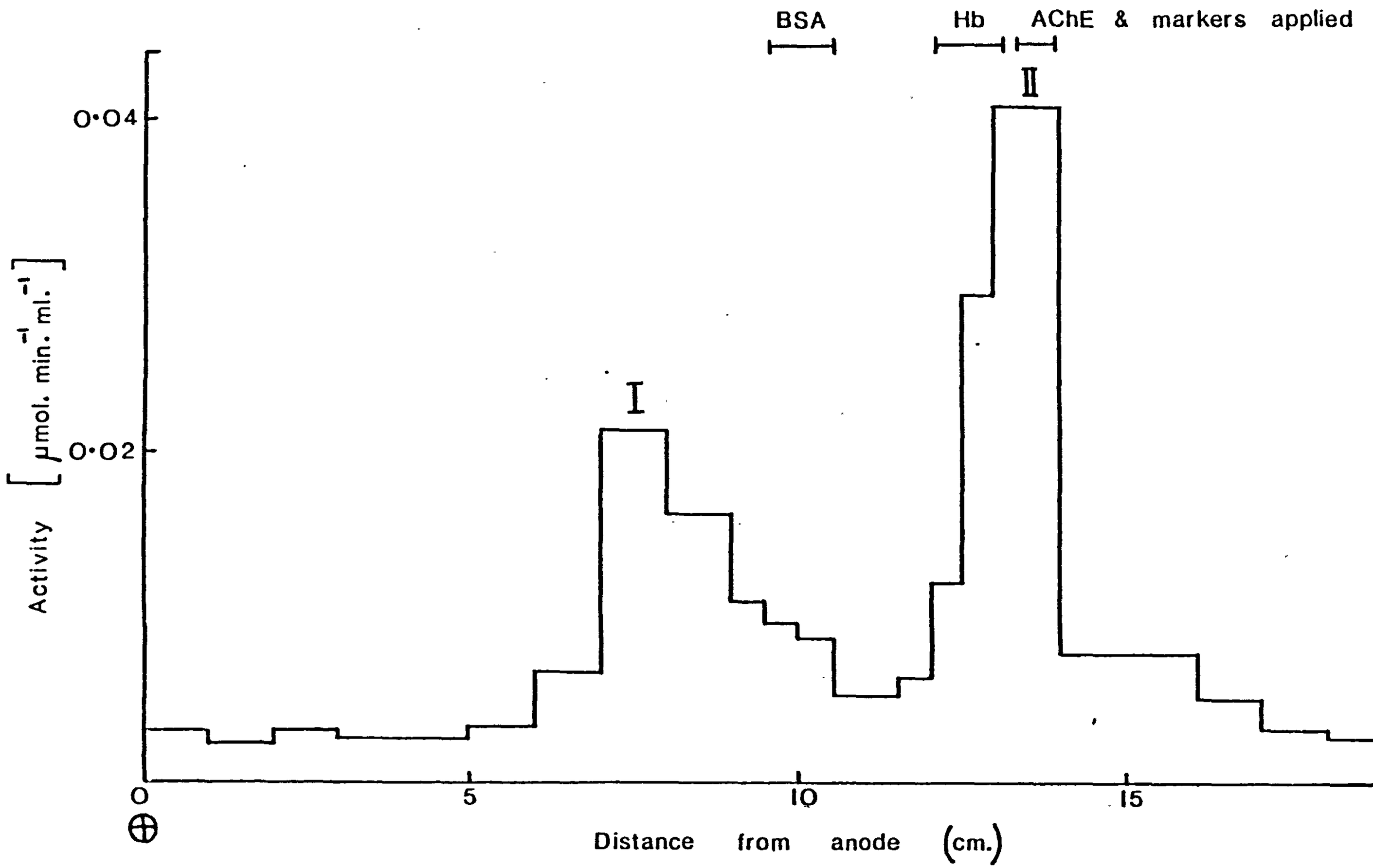
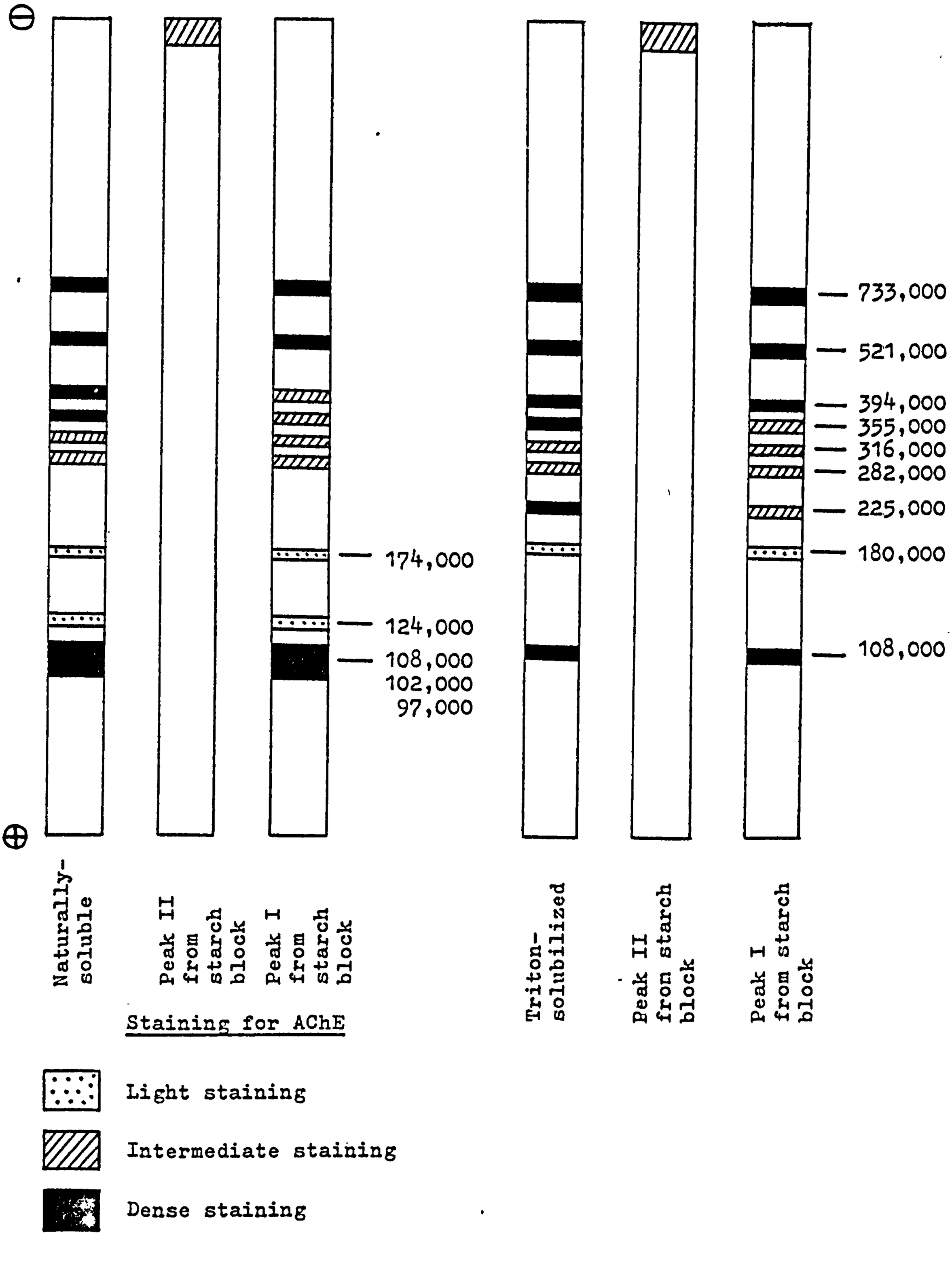


Fig. IV.7 Polyacrylamide Gradient Slab Gel Electrophoresis of Acetylcholinesterase

Electrophoresis was carried out at 100 v. for 24 h.



4. CENTRIFUGATION ON A SUCROSE GRADIENT

When the Triton solubilized AChE was centrifuged on a sucrose gradient containing 1% (w/v) Triton X-100, three peaks of enzyme activity were resolved. These peaks of activity represented three species having sedimentation coefficients of 8.5 S, 16 S and 20 S which corresponded to molecular weights of 155,000; 399,000 and 558,000 respectively. (Fig. IV.8)

The naturally soluble enzyme when centrifuged on a sucrose gradient showed two peaks of enzyme activity in the 6 S and 16 S regions. These sedimentation coefficients corresponded to two species of molecular weights 92,000 and 399,000. (Fig. IV.10)

The peaks of enzyme activity of the Triton solubilized and naturally soluble AChE resolved by starch block electrophoresis were also centrifuged on sucrose gradients. Peak II from both enzyme preparations migrated to the bottom of the gradient suggesting the presence of a very high molecular weight species. Peak I from both preparations gave a single peak of enzyme activity in the 11 S region which corresponded to a species having a molecular weight of 227,000. (Figs. IV.9 and IV.11).

Fig. IV.8 Sucrose Density Gradient Centrifugation of Triton-solubilized Acetylcholinesterase Incorporating 1% Triton X-100 (w/v)

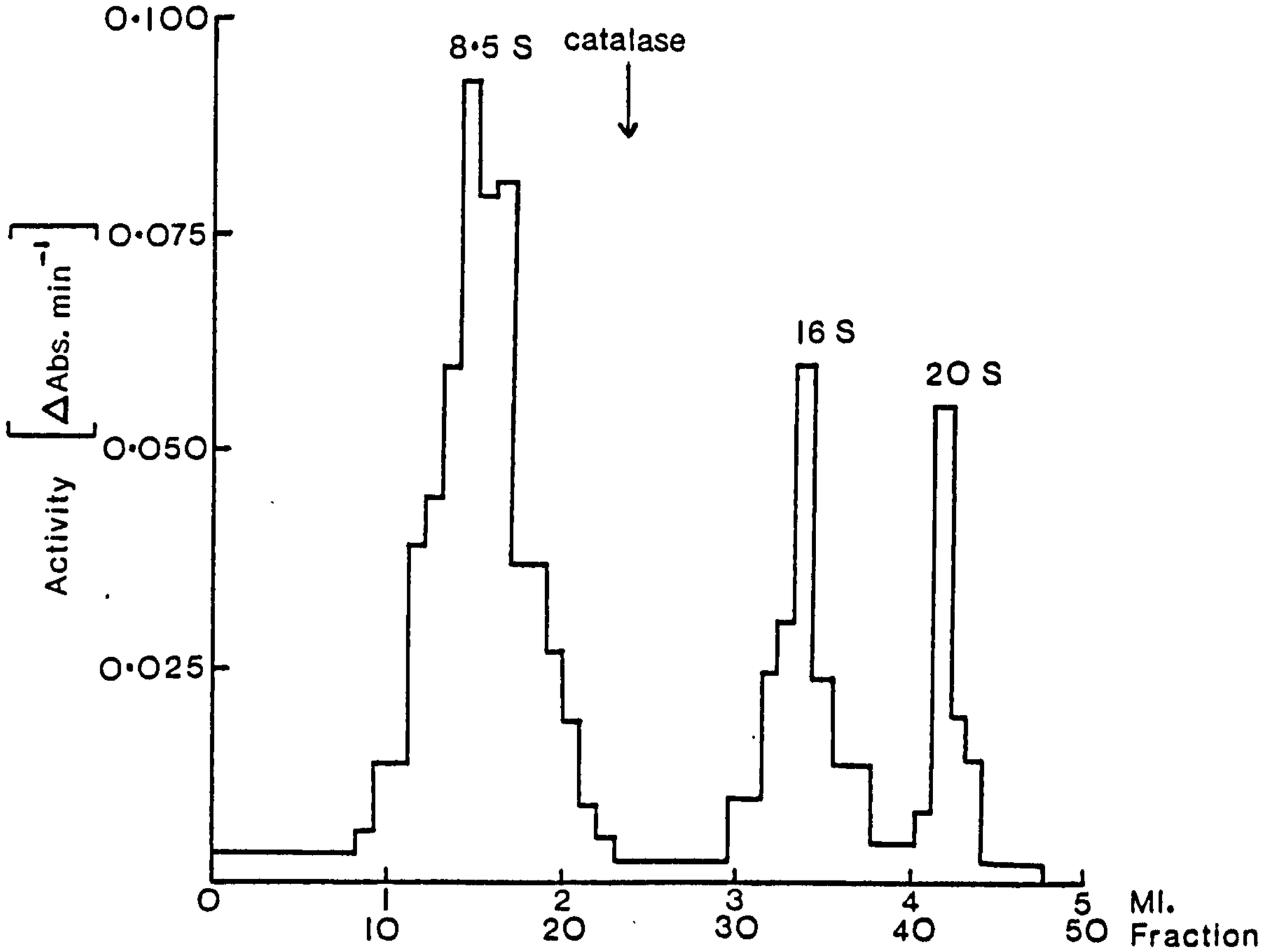


Fig. IV.9 Sucrose Density Gradient Centrifugation of !Peak I' from Starch Block Electrophoresis of Triton solubilized Acetylcholinesterase Incorporating 1% Triton X-100 (w/v)

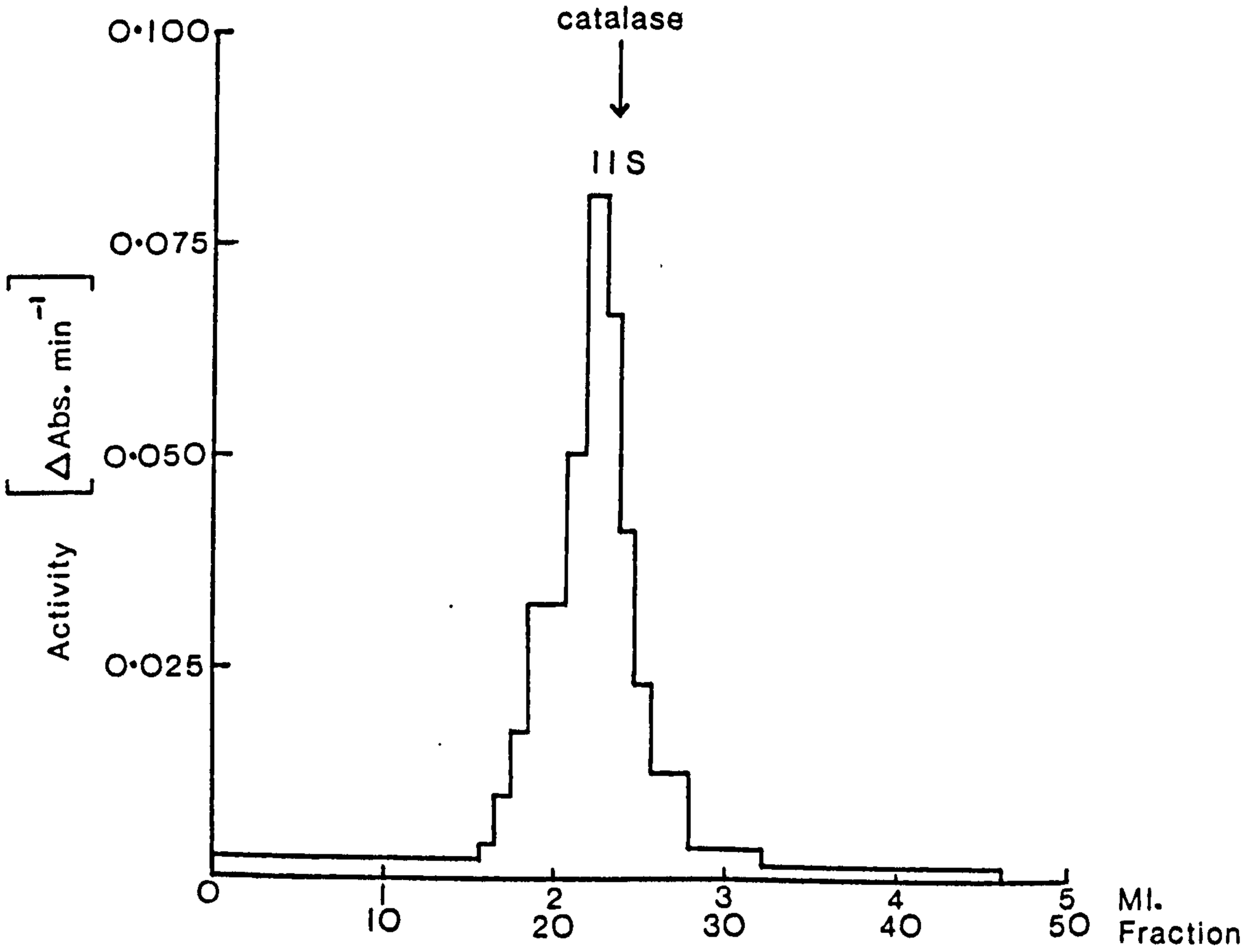


Fig. IV.10 Sucrose Density Gradient Centrifugation of Naturally soluble Acetylcholinesterase

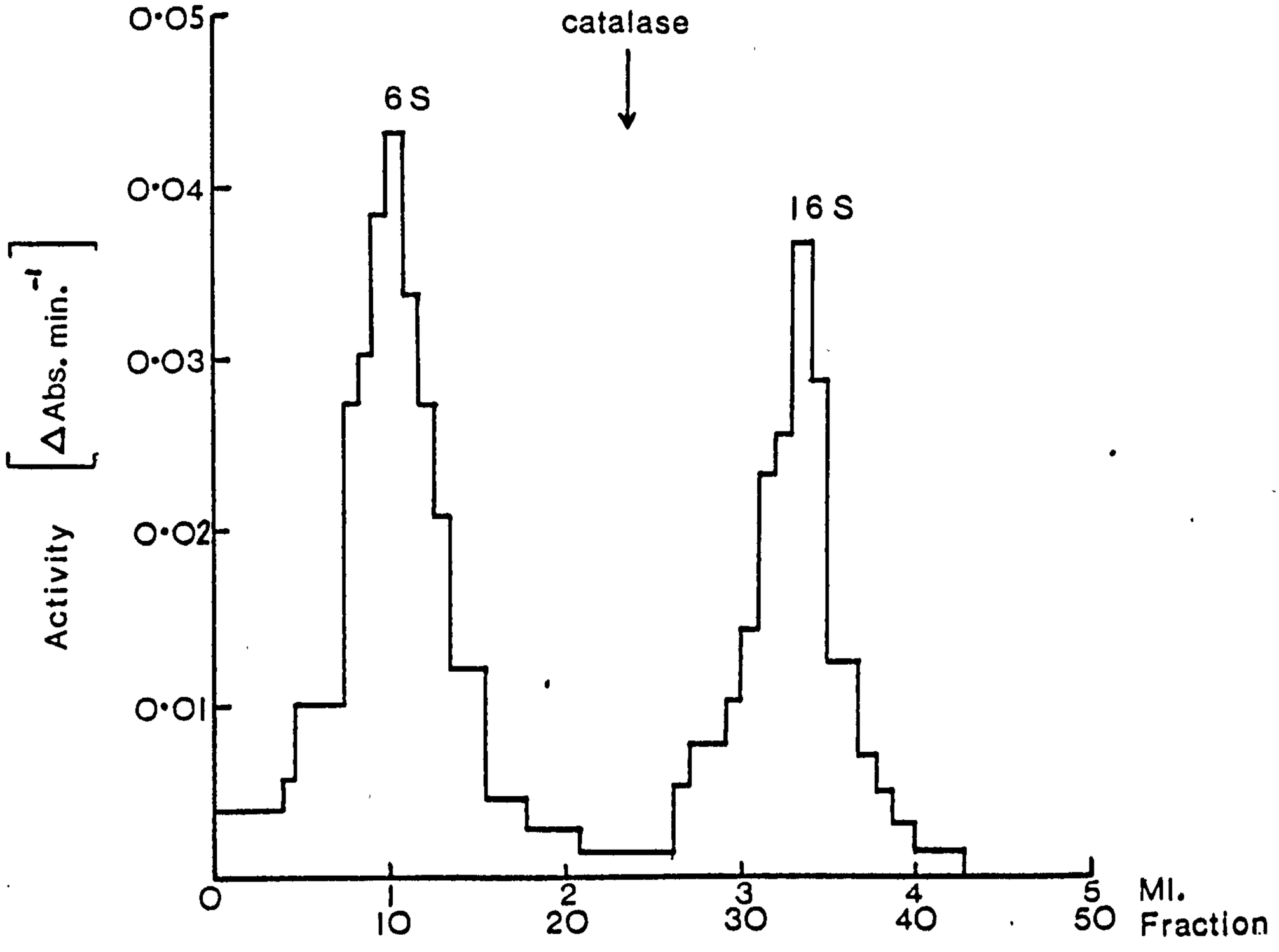
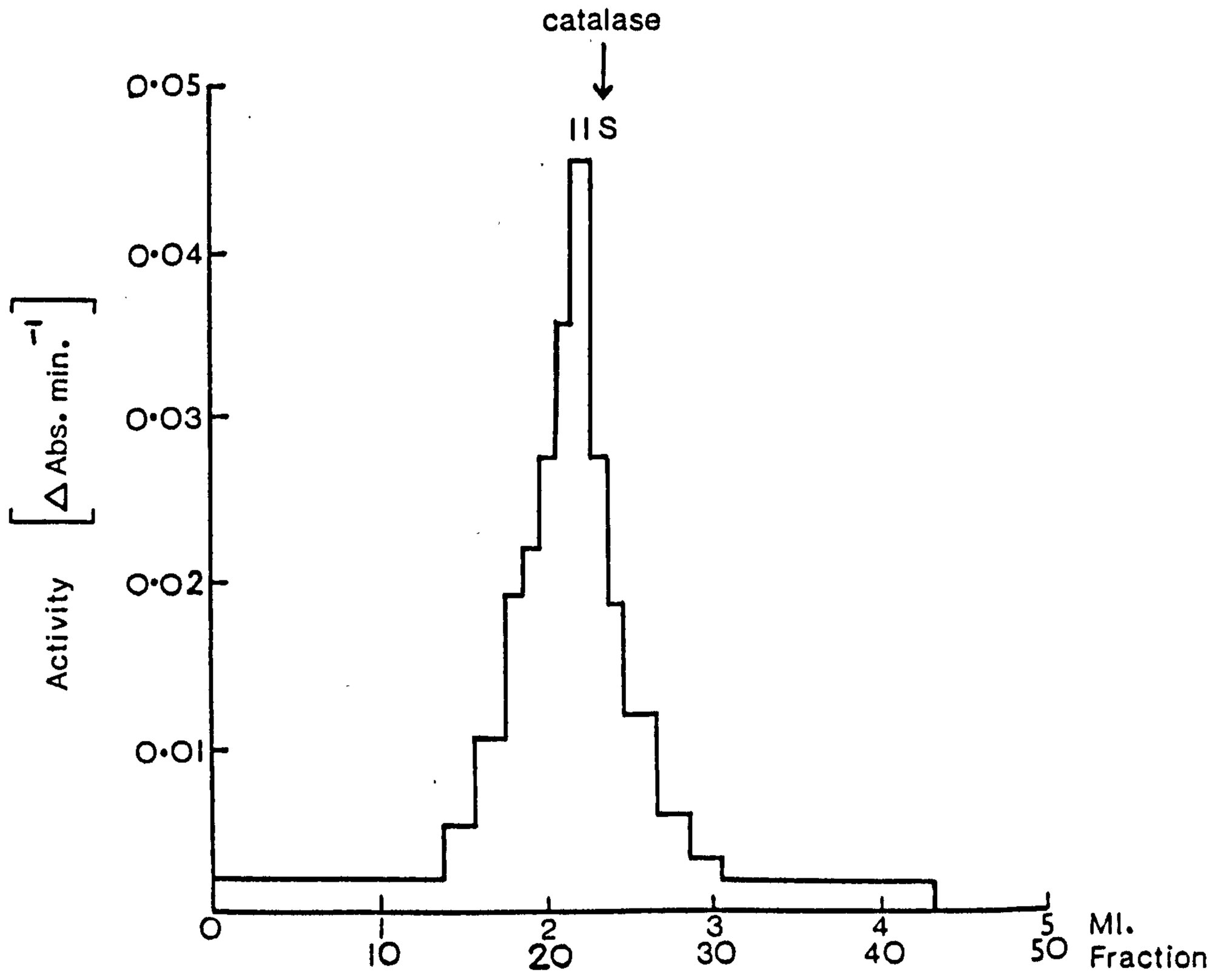


Fig. IV.11 Sucrose Density Gradient Centrifugation of 'Peak I' from Starch Block Electrophoresis of Naturally soluble Acetylcholinesterase



5. THE EFFECT OF ORGANOPHOSPHORUS COMPOUNDS

Inhibition of the enzyme preparations (naturally soluble AChE and Triton solubilized AChE) by organophosphates both in the presence and absence of substrate were carried out so as to establish the type of kinetics that the organophosphate would exhibit towards the enzyme. Such experiments reveal the nature of the organophosphate inhibition, showing it to be either competitive or non-competitive inhibition with regard to the substrate. In turn this would lead to some suggestion regarding the site of action on the enzyme by the organophosphate.

The pseudo-first-order rate constants and bimolecular rate constants (k_i) for inhibition of the naturally soluble and Triton solubilized AChE by organophosphorus compounds, both in the presence and absence of substrate, were determined. This was carried out according to the method described by Main & Iverson (1966) and the accuracies of the constants were computed by the least-squares method of linear regression with a maximum-likelihood programme. Double-reciprocal plots (reciprocal of the velocity of substrate reactions catalysed by the esterase remaining uninhibited versus the reciprocal of the inhibitor concentration) were used to present the data obtained according to the linear form of the equation,

$$k_i = k_p/K_a = \frac{2.3\Delta \log v}{\Delta t}\left(\frac{1}{i} + \frac{1}{K_a}\right) \qquad (1)$$

(Wilson, 1960; Main, 1964), which is shown below:

$$\frac{\Delta t}{2.3\,\Delta \log v} = \frac{1}{ik_i} + \frac{1}{k_p} \qquad (2)$$

where '$2.3\,\Delta \log v/\Delta t$' is considered to be the dependent variable 'y' and 'i' is the independent variable 'x'. (Kitz & Wilson, 1962; Main, 1964; Gold & Fahrney, 1964). Also, k_i represents the bimolecular rate constant; k_p represents the phosphorylation constant; K_a represents the affinity constant; 'v' represents the velocity of the reaction; 't' represents the time and 'i' represents the inhibitor concentration.

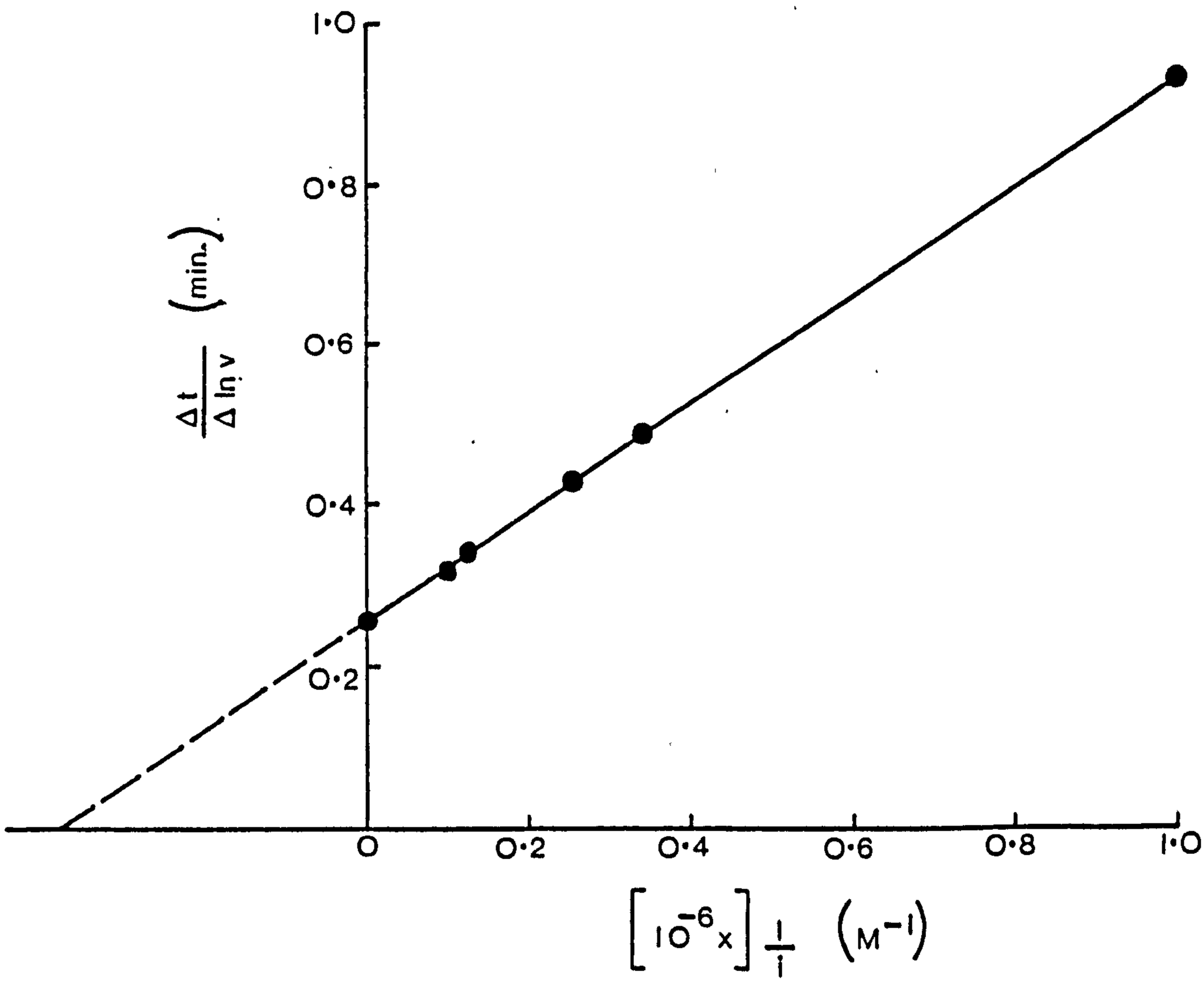
As shown in equation (2), the plot of $\Delta t/2.3\,\Delta \log v$ versus 1/i (Double-reciprocal plot) would give the bimolecular rate constant (k_i). (Fig. IV.12). When substrate is present in the inhibition reaction, the term $(1-\alpha)$ where $\alpha = [S]/(K_m + [S])$, is introduced into the inhibitor concentration variable and so in the presence of substrate the inhibitor concentration becomes $i(1-\alpha)$.

The results obtained for both enzyme preparations showed that the bimolecular rate constants were the same whether or not substrate was present during inhibition of the enzyme. (Tables IV.6 and IV.7). These results therefore suggest that the organophosphates react with the free active centre of the enzyme.

The k_i values of the naturally soluble enzyme were lower than those of the detergent solubilized enzyme for all the inhibitors studied which shows that the naturally soluble enzyme was less susceptible to inhibition than the Triton solubilized enzyme. The Triton solubilized AChE was 2-fold more sensitive to inhibition than the naturally soluble AChE in the presence of malaoxon and this increased to 4-fold in the presence of methyl paraoxon.

The enzyme preparations were also observed to be sensitive to different inhibitors. The naturally soluble AChE was most strongly inhibited by ethyl malaoxon while the Triton solubilized AChE was most strongly inhibited by methyl paraoxon.

Fig. IV.12 Typical Double-reciprocal Plot Showing $\Delta t/\Delta \ln v$ versus $1/i$



The slope of the graph gives the reciprocal of the bimolecular rate constant (k_i).

Table IV.6

Bimolecular rate constants (k_i) for inhibition of Naturally-soluble AChE

Inhibitor	Bimolecular rate constant (k_i) 10^{-4} X k_i (M^{-1}. $min.^{-1}$)	
	In the presence of substrate	In the absence of substrate
Malaoxon	38.0 ± 1.6	36.0 ± 1.8
Ethyl Malaoxon	65.0 ± 4.2	63.5 ± 3.9
Paraoxon	37.0 ± 1.5	38.0 ± 1.7
Isopropyl Paraoxon	11.7 ± 0.42	12.0 ± 0.5
Methyl Paraoxon	50.0 ± 3.8	47.5 ± 3.0

The results show the mean value ± S.E.M. for 4 experiments.

Table IV.7

Bimolecular rate constants (k_i) for inhibition of Triton-solubilized AChE

Inhibitor	Bimolecular rate constant (k_i) 10^{-4} X k_i (M^{-1}. $min.^{-1}$)	
	In the presence of substrate	In the absence of substrate
Malaoxon	88.0 ± 6.0	86.0 ± 5.2
Ethyl Malaoxon	159.0 ± 7.3	160.0 ± 8.0
Paraoxon	110.0 ± 4.0	106.0 ± 3.7
Isopropyl Paraoxon	40.0 ± 2.2	38.4 ± 1.3
Methyl Paraoxon	175.0 ± 7.6	172.6 ± 7.1

The results show the mean value ± S.E.M. for 4 experiments.

SECTION V

DISCUSSION

PORCINE BRAIN ACETYLCHOLINESTERASE

The first aim of the research presented in this thesis was to attempt to find an efficient method for solubilizing the porcine brain acetylcholinesterase. After solubilization of the porcine brain enzyme, the enzyme was purified by affinity chromatography and its molecular forms were examined.

Following the above investigations, attempts were made to determine (i) the relationship of the acetylcholinesterase with the membrane and (ii) the significance of its function in the membrane with respect to the theories of nerve impulse transmission.

1. SOLUBILIZATION

The process of solubilization is usually understood in terms of the inability of the solubilized fraction to form a sediment after centrifugation at 100,000 g. for 1 hour. In addition, the solubilized fraction is expected to show some retardation on a detergent-equilibrated molecular sieve column and to be devoid of detectable membrane structures when examined by electron microscopy (Razin, 1972).

The criteria upon which the method of solubilization of the enzyme was adopted were established according to the following parameters (i) the yield of enzyme, (ii) the preparation time, (iii) the degree of complexity of the solubilization procedure. Among the parameters mentioned, the yield of enzyme was considered to be the principal criterion. Several solubilization procedures were tried, most of which were previously adopted for solubilization of acetylcholinesterase from various species and tissues.

A. Dilute Buffer or Aqueous Media

When porcine brain homogenate was incubated in dilute buffer or aqueous solution, an enzyme yield of between 13% and 17% of the total homogenate activity was consistently obtained (Table III.2). This value for the naturally soluble enzyme is in agreement with the results obtained by several workers (Ho & Ellman, 1969; McIntosh, 1973; Devonshire, 1975; Reavill, 1976). Initially, this soluble AChE was thought to be in equilibrium with the enzyme which was present on the membrane and therefore was not a true soluble fraction. Later, when the 100,000 g. pellet from this preparation was rehomogenised in dilute buffer or aqueous solution, no more enzyme was solubilized, indicating that the naturally soluble AChE was a true soluble fraction. A satisfactory explanation for the origin of the soluble AChE is yet to be found but it is possible that it originates from the membrane enzyme which is constantly being turned over.

B. Chelating Agents

Several groups have used ethylenediaminetetraacetic acid (EDTA) for the solubilization of enzyme from the membrane as EDTA is known to facilitate the removal of proteins from the membrane probably by disrupting the divalent ion bridges which stabilize protein-lipid complexes (Hollunger & Niklasson, 1973; Maddy & Dunn, 1973). Chan *et al* (1972a,b) and Hollunger & Niklasson (1973) have applied EDTA in their solubilization procedure and they obtained between 50% and 70% of the enzyme from the membrane fraction. Under similar experimental conditions as above, it was possible to solubilize between 2.8% and 3.2% of the total homogenate activity (Table III.2). The extremely low yield of enzyme obtained by this

method made it an unsatisfactory procedure for enzyme preparation. However, in the grey matter of porcine brain, solubilization of AChE by this method showed that 49% of the total homogenate activity could be solubilized (Fig. III.5). Chan et al (1972b) reported that 40% of the AChE could be solubilized from bovine brain caudate nucleus by EDTA. These results suggest that the AChE in the white matter of porcine brain is much more firmly bound to the membrane than the enzyme in the grey matter and the enzyme in bovine brain caudate nucleus.

Research on the solubilization of AChE by EDTA was extended by investigators such as Dudai & Silman (1973) and Lerner et al (1972). They showed that when divalent cations such as Ca^{2+} were included in the homogenizing medium, there was a marked decrease in the enzyme activity found in the 100,000 g. supernatant. Hollunger and Niklasson (1973) stressed that this was not an ionic strength effect because equivalent concentrations of monovalent cations such as Na^{+} and K^{+} had considerably less influence on the solubilization of AChE. They also found that when tetracaine was incorporated into the EDTA extraction medium the solubilization of AChE was increased and their explanation was that the tetracaine probably displaced Ca^{2+} from its binding to phospholipids and/or proteins thus reducing the forces binding the AChE to the membrane.

C. Triton X-100

The use of detergents (synthetic detergents, bile salts and saponins) appear to be the most generally useful extraction procedure for the solubilization of proteins which are more strongly bound to the lipid matrix of the membrane (Helenius & Simons, 1975). A delicate balance exists between solubilization and inactivation

of proteins and generally, the inactivation follows the order: nonionic detergents (Lubrol, Triton X-100, Tween 20)<bile salts (cholate, deoxycholate)< anionic detergents (dodecyl sulphate) and cationic detergents (cetyltrimethylammonium). The degree of solubilization also depends on the relative concentrations of membrane and detergent (Coleman, 1973).

The solubilization of AChE by detergents has been adopted as a successful enzyme preparation procedure by several workers (Ho & Ellman, 1969; Wright & Plummer, 1972; McIntosh, 1973; Reavill, 1976). In these studies, the method of Ho & Ellman (1969) with slight modifications, was followed. From the results obtained (Table III.2) it is conclusive that Triton X-100 was not an effective solubilizing agent for the white matter AChE as between 16% and 18% of the total homogenate activity was obtained. In contrast, McIntosh (1973) and Reavill (1976) obtained an enzyme yield of 56% of the total homogenate activity for the solubilization of grey matter AChE from porcine brain (Fig. III.5) while Ho & Ellman (1969) obtained a yield of 85% for the solubilization of rat brain AChE. The relatively low yield obtained could suggest that the white matter AChE is strongly associated with the lipid matrix of the membrane and hence Triton X-100 which is a mild detergent is unable to dissociate the enzyme successfully from the membrane.

When increasing concentrations of Triton were added to the extraction medium, there was an increase in the amount of enzyme solubilized with a maximal effect at 2% (w/v) Triton X-100. (Fig. III.1). This observation is similar to the results obtained by McIntosh (1973). In addition, the results obtained for the experiments to investigate the effect of KCl on Triton solubilization

of brain AChE were similar to those obtained by Wright & Plummer (1970, 1972) and McIntosh (1973) (Fig. III.2). Variations in the values obtained may occur as it is well known that commercial preparations of detergents vary quite significantly with different amounts of additives and water being present. Secondly, the number of ethylene oxide groups per molecule of detergent stated by the manufacturer is only a mean value as Triton X-100 which is a polyoxyethylene p-t-octyl phenol has polydisperse polyoxyethylene head groups due to the statistical polymerization of ethylene oxide.

The use of KCl and Triton X-100 combination in the extraction medium marginally increased the solubilization of AChE. An explanation for this effect is that KCl probably weakens the electrostatic bonds which bind membrane proteins and hence facilitates solubilization of the enzyme. The appearance of lipid in the 100,000 g. supernatant may be significant. Generally, the enzyme is probably in a partly lipid environment which could influence some control over its activity and removal of this lipid might remove the control and hence alter the kinetic features of the enzyme. However, no conclusive statement can be drawn from the experiments described in this thesis but Morero et al (1972) reported that lipid had significant effects on allosteric properties of erythrocyte AChE.

Several reports have appeared in the literature on the effect of Triton X-100 on enzyme activity. Jackson and Aprison (1966b), Harwood and Hawthorne (1969) and Srinivasan et al (1972) found that Triton X-100 activated AChE by enhancing its activity apart from solubilizing it, while Fiszer and De Robertis (1967) found no activation and Gitler et al (1967) found some inhibition of erythrocyte

AChE at low Triton concentrations. Crone (1971) suggested that the activation associated with solubilization depends largely on the method of preparation of the tissue fraction used as the source of enzyme. Raaijmakers (1978) found that freezing brain tissue before homogenization is an important factor determining the degree of activation of AChE by Triton X-100. He suggested that this led to an increased occlusion of AChE activity in the homogenate which caused the major part of the activation of AChE by Triton X-100. Raaijmakers also investigated the effect of Triton X-100 on soluble and membrane-bound fractions of AChE and he found that there was no activation of AChE in the soluble fraction but activation occurred in the membrane-bound fraction. Skangiel-Kramska and Niemierko (1975), working with peripheral nervous tissue also found that Triton X-100 did not increase the activity of soluble AChE but it had an activating effect on the crude homogenate. These observations suggest that the activating effect of Triton X-100 depends on the membrane-bound state of the AChE molecules. In this thesis, the results obtained show that there is no such activation of AChE in all preparations which were incubated with the detergent.

The advantages of using Triton X-100 as a solubilizing agent are that it does not induce conformational changes in proteins leading to a loss of their biological properties (Meunier et al, 1972; Rubin & Tzagoloff, 1973), and it also appears to be very inefficient in disrupting protein-protein interactions (Helenius & Simons, 1975), although it has been reported that the quaternary structure of some proteins (eg. haemocyanin,alkaline phosphatase and haemoglobin) was disrupted (Helenius & Simons, 1972). Utermann and Simons (1974) have demonstrated that Triton X-100

might be bound to the hydrophobic domain of glycoproteins and not to their hydrophilic parts. Since AChE has been reported to be a glycoprotein (Powell et al, 1973) and also possessing hydrophobic areas on its surface (Steinberg et al, 1975) it is possible that some detergent binds to the enzyme.

D. Lysolecithin

Lysolecithin has been shown to cause fusion of hen erythrocytes at low concentrations, i.e. <0.6 mmol/l (Poole et al, 1970). However, at higher concentrations of the surfactant, lysis of the membrane occurs. According to the results published by Marples et al (1959) and McArdle et al (1960), incubation of brain slices with 12 mmol/l lysolecithin released 45% of the AChE into the surrounding medium. When identical experimental conditions were established, incubation of white matter with lysolecithin for 30 min. resulted in the release of between 30% and 36% of the total homogenate activity. This value is much lower than that obtained by McArdle et al (1960) who reported a value of 80% and the variation in results obtained could be attributed to the different source of tissue. Since lysolecithin treatment was not very efficient in the solubilization of white matter AChE and in addition to the expense of pure lysolecithin, this method was abandoned as a routine solubilization procedure.

Haydon and Taylor (1963) have suggested that surfactants may act as 'wedges' which destroy the natural orientation of the lipid bilayer. Lucy (1960) has also proposed a possible mode of action for lysolecithin in which the incorporation of the 'wedge-shaped' lysolecithin is thought to produce a transition from a bimolecular leaflet to a micellar structure. The effectiveness of lysolecithin

as a solubilizing agent depends on its ability to form micelles with membrane lipids and it has been reported that treatment of myelin with lysolecithin yields a complex protein-lipid-lysolecithin micelle (Gent et al, 1964).

E. Bile Salts

Bile salts have been successfully used to solubilize many membrane proteins without loss of biological activity (Spatz & Strittmatter, 1971; Meunier et al, 1972; Snary et al, 1974).

When either sodium cholate or sodium deoxycholate prepared in 0.05 mol/l Tris buffer, pH 7.5, were used as the extraction medium, enzyme yields of between 16.5% and 20.5%, and between 66% and 72% of the total homogenate activity, respectively, were obtained (Table III.2). From these results, it can be seen that sodium cholate (trihydroxy bile salt) is not as effective as sodium deoxycholate (dihydroxy bile salt) when used as a solubilization agent for white matter.

In the case of sodium cholate, increasing the concentration of the bile salt in the extraction medium increased the amount of AChE solubilized with a maximal effect at 1% (w/v) sodium cholate (Fig. III.3). However, for soidum deoxycholate, an increase in the bile salt concentration up to 0.3% (w/v) resulted in an increase in the amount of enzyme solubilized, but above this concentration there was slight inhibition (3% - 12%) of the enzyme activity (Fig. III.4). From the results obtained, (Table III.1, Table III.2) sodium decxycholate is found to be the most efficient solubilizing agent for white matter and using this detergent between 66% and 72% of the total homogenate activity is solubilized. Since bile salts are known to be strong detergents and the results obtained show that

solubilization with this detergent is very efficient, it is tempting to suggest that AChE in the white matter is bound tightly to the membrane.

The physical properties of bile salts have been investigated in many laboratories because of their physiological role in lipid absorption in the gut (Small, 1970; Carey & Small, 1972). In the bile salts, the polar groups are distributed in different parts of the molecule and there is no well-defined 'head group' (Fig. II.6). The hydroxyl groups are located on one side of the rigid cyclopenthenophenantrene ring structure and the terminal ionic group is situated at the end of a short flexible branched aliphatic chain. The bean shaped molecule thus possesses a polar and an apolar face, and consequently orientates itself 'flat' on air/water interphases. In water above a critical concentration, bile salts form small aggregates (from dimers to octamers) in which the molecules lie back to back. At higher counter ion concentrations larger aggregates may form (secondary micelles), in contrast to the smaller 'primary micelles'. The 'secondary micelles' are probably made up of 'primary micelles' bound to each other by polar interactions forming globular clusters. The critical micellar concentration (CMC, i.e. the concentration at which aggregation starts) and the aggregation number of trihydroxy bile salts are resistant to the counter ion concentration whereas with dihydroxy bile salts the aggregation number increases and the CMC decreases with increasing counter ion concentration (Carey & Small, 1972).

The properties of bile salts which possess carboxyl groups are very dependent on pH (Small, 1968; Carey & Small, 1972). When the pH is lowered to values approaching the pK_a, bile acid is formed

which is insoluble in water. The acid can be solubilized to some extent by the bile salt micelles present but when these are saturated, the acid begins to precipitate. Sodium cholate precipitates at pH 6.5 and sodium deoxycholate at 6.9. Hence in the studies relating to solubilization of AChE by these bile salts, a pH of 7.5 was maintained. In the case of deoxycholate, the occurrence of the acid form is accompanied by a dramatic increase in the micellar size. At a pH just above the precipitation limit deoxycholate forms a gel. As a result of these findings, it is advisable to use slightly alkaline pH or conjugated bile salts in membrane studies.

In contrast to denaturing detergents, deoxycholate appears to interact predominantly with proteins which are bound to the membrane lipids by hydrophobic interactions. Usually the binding of these detergents does not lead to major conformational changes of the protein and loss of activity. The detergent molecules are thought to bind to the hydrophobic region and not to the hydrophilic parts of the amphiphilic protein. It is still uncertain whether the detergent molecules are all bound to individual sites or whether only a part of the bound molecules would interact directly with the protein and the rest bind cooperatively to form a micelle-like region on the protein surface. Robinson et al (1974) and Helenius & Simons (1975) suggest that a micelle-like interaction is more likely to occur. The important feature of this scheme is that the environment around the hydrophobic region of the protein remains apolar and that around the hydrophilic parts aqueous throughout the solubilization process. Hence the orientation of the protein in two different phases is preserved during solubilization and in many cases the protein-bound detergent mimics the lipid environment in

the membrane sufficiently well to support continued activity of the protein. Such gentle two-phase extraction is difficult to achieve for amphiphilic proteins using any other solubilization method. This scheme is supported by results obtained mainly from studies of the SF virus membrane proteins and cytochrome b_5 (Utermann & Simons, 1974; Robinson et al, 1974).

Although the bile salts do not usually function as protein denaturants, there are some membrane proteins which lose their biological activities when solubilized with these detergents, e.g. $(Na^+ + K^+)$ - ATPase, Ca^{2+} - ATPase, cytochrome oxidase, glucose-6-phosphatase and adenylate cyclase. This may be due to (i) these proteins being less resistant to denaturation (i.e. the active conformation of the protein may be destroyed by the disruption of essential protein-lipid or protein-protein interactions during solubilization), (ii) detergent binding to the functional sites on the proteins, (iii) removal of cofactors necessary for activity, (iv) inactivation because of 'assay problems' - it is difficult to ascertain whether changes in activity result from the direct action of detergent on the enzyme molecule or on the membrane environment around the enzyme. A common feature for the denaturing detergents appears to be a combination of a charged head group and a flexible apolar tail. The nature of the charged head group and the length of the detergent alkyl tail are important and influence both the critical concentration needed to induce cooperative binding and the resulting conformational change (Steinhardt & Reynolds, 1969; Tanford, 1973). Deoxycholate and Triton X-100 have rigid and bulky apolar moieties which probably do not penetrate the crevices of the protein surfaces as efficiently as the flexible alkyl chains. It

is possible that both deoxycholate and Triton X-100 could induce cooperative binding and denaturation if high enough concentrations of the free monomeric form of the detergent could be reached. However, this is not possible because of micelle formation, and the monomer concentration is limited approximately to the CMC of the detergent. In addition, the effect of detergents on membrane enzymes is sometimes biphasic; activation is observed at low detergent concentrations and inhibition at higher concentrations.

2. KINETICS

Kinetic analysis of the enzyme was not intensively investigated because it was not essential for the principal objectives of this study. In addition, purified enzyme preparations are necessary to obtain meaningful results.

The pH dependence and substrate dependence of sodium deoxycholate solubilized acetylcholinesterase from white matter of porcine brain (Figs. II.2 and II.3) were similar to the results obtained for grey matter of porcine brain (McIntosh, 1973). Brain AChE from other species also showed similar results to those obtained in this study (Jackson & Aprison, 1966a; Chan et al, 1972b). Studies on the membrane-bound AChE showed some irregularity in the pH-activity profile and this can be attributed to micro-pH effects on the membrane surface which was reported by Silman and Karlin (1967).

The specificity of the naturally soluble and sodium deoxycholate solubilized enzymes are very similar and under normal assay conditions, is that of a specific AChE since maximum activity was obtained with acetylcholine and acetylthiocholine (Table III.3).

Also, the hydrolysis of acetyl-β-methylcholine and inhibition of the enzyme at high substrate concentration both confirm this classification. The low hydrolysis of butyrylcholine suggests the presence of a cholinesterase, as was observed in bovine brain (Brestkin & Pevzner, 1971). When a comparison of the specificity of the grey matter and white matter AChE from porcine brain was made (Table III.3), it was observed that they were rather similar.

The Michaelis constants for the white matter AChE from porcine brain (Table III.4) were similar to those from other species (80 - 140 μmol/l) (Jackson & Aprison, 1966a; Ho & Ellman, 1969; Chan et al, 1972b). The differences in K_m values of solubilized enzyme preparations could probably be due to enzyme modification during solubilization but more extensive research is required to reach plausible explanations. The higher K_m value obtained for the soluble enzyme may be due to the presence of a non-specific esterase which could hydrolyze acetylcholine.

3. PURIFICATION OF THE ENZYME

Purification is a very essential process which should be carried out prior to the study of the properties of any biological molecule. It is necessary to purify biological molecules so as to prevent the occurrence of artefacts which could arise from contaminating material.

In our laboratory, porcine brain AChE had previously been partially purified by hydrophobic affinity chromatography (McIntosh, 1973) using a hydrophobic affinity column based on a method developed by Yon (1972). This affinity column was not very efficient in the purification of enzyme as it had limited specificity

for the enzyme, due to the AChE being eluted with different combinations of salt and/or Triton X-100. Therefore in attempts to purify the porcine brain enzyme, Reavill (1976) adopted the procedure of affinity chromatography in which several ligands specific for AChE were used. The affinity columns which were used included the (i) MAP-agarose column (the ligand MAP is N-methyl-3-aminopyridinium iodide), (ii) ε-aminocaproyl-PTA-agarose column (the ligand ε-aminocaproyl-PTA is [N-(ε-aminocaproyl)-p-aminophenyl] trimethylammonium bromide hydrobromide), and (iii) MAC-agarose column (the ligand MAC is [1-methyl-9-(N^{β}-ε-aminocaproyl)-β-aminopropylamino] acridinium bromide hydrobromide). Among these affinity columns used, the MAC-agarose column gave the best enzyme yield and a good degree of purification and so in this study, the MAC-agarose column was used.

The MAC-agarose column was originally developed by Dudai and Silman (1974a) for the purification of an aggregated species of AChE from electric eel. This column was also found to be very successful for the purification of porcine brain enzyme (Reavill, 1976).

The results showed that the MAC-agarose was extremely successful at binding the AChE and up to 2 of the crude enzyme preparation could be run through the column without any of the enzyme being lost in the eluate. This indicates that the enzyme was binding to at least one site on the affinity column.

The AChE was eluted from the affinity chromatography column with decamethonium bromide and after dialysis, it was found to have a purification of 300-fold. The enzyme yield from the decamethonium peak was 24% and the specific activity of this purified

AChE was estimated to be 18.63 μmol.min.$^{-1}$mg.$^{-1}$ protein. The yield of enzyme obtained was much lower than that obtained by Reavill (44%) and Dudai et al (1972b) (50%). Dudai et al (1972b), however, eluted the electric eel AChE from the MAC-agarose column in the presence of high ionic strength NaCl (1 mol/l) instead of just using decamethonium bromide. The relatively low yield of enzyme obtained for the white matter AChE as compared with that for the grey matter AChE from porcine brain could probably be due to the different degree in binding to the column by the enzyme. The difference between the results obtained by Dudai et al (1972b) may be due to different sources of enzyme.

Since a second peak of enzyme activity (Peak II) (Fig. III.6) was eluted from the column with high ionic strength NaCl (1 mol/l), following the inhibitor peak, this suggests that there was some AChE that had bound to the column non-biospecifically, probably by electrostatic interactions. Hydrophobic interactions may also arise from the macroreticular matrix of the Sepharose. A purification of 200-fold and a specific activity of 12.64 μmol.min^{-1}mg.$^{-1}$ protein was obatined for the enzyme eluted from Peak II. Since a purer form of the enzyme was obtained from the decamethonium bromide elution, this enzyme (Peak I) was used for subsequent studies.

4. MOLECULAR FORMS OF BRAIN ACETYLCHOLINESTERASE

During solubilization of any membrane-bound protein it should be borne in mind that the extraction of the protein from its natural environment may change its biological properties to some extent. Depending on the method of solubilization, AChE can be

isolated from various sources in a number of multiple molecular forms which differ in molecular weights, Stokes radii, sedimentation coefficients, and isoelectric properties.

Maddy & Dunn (1973) and Nachmansohn (1975) have both suggested that the variation in molecular weights are probably due to the methods and conditions of solubilization of the enzyme and also to the various degrees of aggregation which arise from different treatments. Therefore it is essential to consider these possibilities when studying the multiple molecular forms of AChE from porcine brain as well as rat muscle. A possible solution to avoid these anomalies is to solubilize the enzyme using different procedures and then resolve and characterize the molecular forms using different techniques. If molecular forms which were solubilized by different methods and resolved by different techniques were similar, then it would be reasonable to assume that the molecular species obtained were not artefacts of preparation.

The techniques used for the separation of molecular species in this study include starch block electrophoresis, polyacrylamide gel electrophoresis and sucrose density gradient centrifugation.

A. Starch Block Electrophoresis

Starch block electrophoresis is a semi-preparative procedure for enzyme purification and although it is a rather laborious method, it can separate molecular forms according to their charge.

The sodium deoxycholate solubilized AChE and the naturally soluble AChE were both resolved into one major peak of enzyme activity. However, the naturally soluble enzyme appeared to have a slightly higher negative mobility than the bile salt solubilized enzyme (Figs. III.7 and III.8).

The affinity chromatography purified enzyme showed the same characteristics as the sodium deoxycholate solubilized enzyme in its elution profile.

In the starch block electrophoresis studies, the results show that for all the enzyme preparations studied, one major peak of enzyme activity was resolved. However, in the separation of molecular species using polyacrylamide gel electrophoresis (which will be discussed later on), several bands of enzyme activity were observed indicating several forms of the enzyme being present. This may be due to the molecular species having very similar charges and hence they are resolved into a single peak by starch block electrophoresis or alternatively, the method of electrophoresis on a starch block is not sensitive enough to resolve the molecular species present. The more likely explanation seems to be that the range of molecular forms observed do not arise from differences in their charges, suggesting that differences in the size or molecular weight of species may be the criterion.

B. Polyacrylamide Gel Electrophoresis

(i) Polyacrylamide rods Electrophoresis on polyacrylamide rods (7% polyacrylamide gel) enables the separation of high molecular weight substances such as proteins and also offers a method of separation for molecular species of an enzyme.

The electrophoretic patterns for the enzyme preparations is shown in Fig. III.9. The figure shows that several similar molecular species are present in the naturally soluble, the sodium deoxycholate solubilized, and the affinity chromatography purified enzyme preparations. The naturally soluble and sodium deoxycholate solubilized enzymes both showed five bands of enzyme activity

indicating the presence of five molecular species. Since this method does not tell us much about the molecular weight of the molecular species, it can only be concluded that a fairly wide range of molecular weights was present in the naturally soluble and sodium deoxycholate solubilized enzymes. The affinity chromatography purified enzyme, however, showed two bands of enzyme activity, one representing a relatively low molecular weight species and the other a high molecular weight species (probably due to aggregation phenomena).

Using this electrophoresis procedure, McIntosh (1973) demonstrated that charge differences in the molecular species were not the only factors for resolution of the species because when the pore size of the polyacrylamide was increased and the electrophoresis time kept the same, protein migration increased but a reduction in species from two to one occurred. McIntosh also observed that when the run was increased to 3 h., two protein bands were again observed. The possible explanations for this phenomenon put forward by McIntosh are (i) either aggregation-disaggregation phenomena occur during electrophoresis, or, more likely, (ii) after 1½ h. run in an increased pore size gel, differences in molecular weight are exactly counterbalanced by charge differences, thus giving an apparent single molecular species.

The similar AChE staining patterns observed for the different preparations studied seem to indicate that the solubilization methods used for the preparation of enzyme did not produce any artefacts.

(ii) Polyacrylamide gradient slabs The reliability of molecular weight determination by gradient gel electrophoresis depends on the

extent to which different proteins comply to the relationship between molecular weight and migration behaviour. A linear relationship between the migration distances and the $\log_{10}$ Molecular weight was obtained for a range of standard proteins (Fig. II.10).

The principle of gradient gel electrophoresis is that proteins migrate through progressively smaller pores, the sizes of which are regulated by gel concentrations, until they reach the point where the size of pore restricts their passage (Slater, 1969). Thus, separation by this technique is related to the size of molecules although in the earlier stages of electrophoresis, separation is based upon both charge and size. The explanation for this is that at the end of a long run (>12 h.) molecular sieving predominates and smaller molecules move further (Margolis & Kenrick, 1968).

There are several advantages for using this technique. Firstly, several proteins may be run side by side on the same gel, thus allowing direct comparison of several proteins; secondly, inaccuracies caused by differences in gel composition may be eliminated; and finally, enzyme species which are present in low concentration and low purity may be resolved adequately, as an alternative to the more accurate sucrose gradient sedimentation technique. In the present study, the time, gel concentration gradient and voltage gradient have all been standardized within a reasonable accuracy.

A number of bands of AChE activity (4 - 5 bands) were obtained for the enzyme preparations using this very sensitive separation technique. The AChE staining pattern for the molecular species are shown in Fig. III.10. From the electrophoretograms, the predominate molecular forms which appear to be present in the different enzyme preparations are species having molecular weights

of 120,000 daltons and 260,000 daltons. These molecular weights are fairly similar to those obtained by sucrose gradient centrifugation, which are 209,000 daltons and 215,000 daltons. In addition, these molecular weights also agree with those obtained by McIntosh and Plummer (1973) who obtained values of between 245,000 daltons and 288,000 daltons for the enzyme from the cortex of porcine brain . Several workers also obtained molecular forms from various sources whose molecular weights were found to range from 102,000 - 134,000 daltons (for protomers) and 224,000 - 260,000 daltons (for oligomers) (Table I.2).

Leuzinger et al (1968) suggested that the native enzyme in the electric eel was a tetramer with a molecular weight of 260,000 daltons and that it is composed of subunits with a M.W. of 64,000 daltons. However, Dudai and Silman (1972) found two types of subunit of M.W. 59,000 daltons and 85,000 daltons for the same species (electric eel) and they suggested that the smaller subunit was an autolysis product from the larger subunit which in turn associated to form a tetramer of M.W. 320,000 - 350,000 daltons. The lowest molecular weight obtained for the different enzyme preparations in this study was 120,000 daltons, which was higher than the values obtained by McIntosh and Plummer (1973) and Reavill (1976), who obtained 60,000 daltons and 68,000 daltons respectively, for the pig brain enzyme. Reavill (1976) suggested that the monomer for the porcine brain enzyme could have a molecular weight of 68,000 daltons and that the species with a M.W. of 250,000 daltons probably was a tetramer composed of 4 of these subunits. From the results obtained in this study, it is tempting to speculate that the species having a M.W. of 120,000 daltons is a dimer and that

the species with a M.W. of 260,000 daltons is a tetramer. The band of enzyme activity in the sodium deoxycholate solubilized AChE (including the peak obtained from starch block electrophoresis), and in the affinity chromatography purified AChE, corresponding to a M.W. of 181,000 daltons could have been a trimer, on the assumption that the monomer had a M.W. of 60,000 daltons.

An interesting observation is that the range of molecular weights for the species in the sodium deoxycholate solubilized AChE is lower than that obtained for the naturally soluble AChE, this being 120,000 - 422,000 daltons and 120,000 - 661,000 daltons respectively. Kremzner and Fei (1971) had proposed the existence of a protein of membrane origin with a M.W. of 150,000 daltons which was responsible for the aggregation of AChE. Therefore a possible explanation for the presence of smaller species after electrophoresis of the sodium deoxycholate solubilized enzyme may be that the 'aggregating factor' is removed, thus allowing the development of smaller molecular forms. Similarly, for the affinity chromatography purified enzyme, a still lower range of molecular weights for the molecular species is observed, with molecular weights ranging from 120,000 - 260,000 daltons. A similar explanation for this observation is that the 'aggregating factor' is probably removed during affinity chromatography and also during electrophoresis, thus allowing a range of smaller molecular forms to exist.

The peak from starch block electrophoresis of sodium deoxycholate solubilized AChE showed an identical enzyme staining pattern on gradient gels as the crude sodium deoxycholate solubilized AChE. This indicates that the differences in multiple

molecular forms arise from the differences in molecular weight rather than charge.

The affinity chromatography purified enzyme showed a range of species (5) with molecular weights ranging from 120,000 - 260,000 daltons. The predominate species were those with molecular weights of 120,000, 181,000 and 260,000 daltons and if it was assumed that the monomer had a M.W. of 60,000 daltons, then these species would correspond to a dimer, trimer and tetramer, respectively. The enzyme bands were also observed to be sharper, indicating better resolution and this may be due to the removal of contaminating proteins by affinity chromatography.

C. Sucrose Density Gradient Centrifugation

This technique is very useful for separating molecular forms of an enzyme and it provides a good estimation of the molecular weights of enzyme species.

Initially, sucrose gradient centrifugation was intended for the separation of molecular forms of AChE in sufficient quantities for further study. Unfortunately, this was not possible as it was demonstrated that when large amounts of protein were applied to the sucrose gradient and centrifuged, the linear relationship between the migration of protein down the tube and the sedimentation constant of that protein was abolished (Reavill, 1976). Steensgaard et al (1975) also observed this effect and they showed that when the sucrose gradients were overloaded with protein, the migrating zone mass centres were dislocated, zone shapes were altered and even individual protein zones were split. However, the linear relationship mentioned is retained if the amount of protein applied to the sucrose gradient did not exceed 50 mg. per gradient (Reavill, 1976).

This agrees with the observation of Martin & Ames (1961) and also avoids the problem of overloading the gradient. Therefore the technique of sucrose gradient centrifugation was considered suitable for the separation of molecular forms of AChE when the precautions mentioned were noted.

The sodium deoxycholate solubilized AChE when run on sucrose gradients migrated to the bottom of the tube, indicating the presence of a highly aggregated species. When 0.2% (w/v) sodium deoxycholate was incorporated into the sucrose gradient, the aggregation was abolished and the enzyme was resolved into a single peak of activity corresponding to a 10.6 S species with a M.W. of about 215,000 (Fig. III.12). Viana et al (1974) also obtained a molecular species with a very similar M.W. of 219,000. This molecular form obtained probably resembles the globular tetrameric enzyme from the electric eel which has a sedimentation coefficient of 11.1 S and a corresponding M.W. of 260,000. According to the nomenclature of Massoulié (Massoulié & Rieger, 1969), this form is designated G_p (Table I.3). Reavill (1976) reported that the 11 S Triton solubilized AChE from the cortex of porcine brain was also found to aggregate. Since both the sodium deoxycholate solubilized AChE and the Triton solubilized AChE were extracted with detergent to bring them into solution, it is possible that the solubilization process may have caused a tendency to aggregate in the solubilized AChE (Maddy & Dunn, 1973).

The naturally soluble AChE was resolved into a single peak of enzyme activity when centrifuged on sucrose gradients, corresponding to a 10.4 S species with a M.W. of 209,000 (Fig. III.13). From these results, it may be concluded that the major species

found in both naturally soluble AChE and sodium deoxycholate solubilized AChE are very similar. Reavill (1976) obtained a major molecular species having a sedimentation coefficient of 11 - 12 S (M.W. 240,000) for the naturally soluble AChE in the grey matter of porcine brain. Therefore the major molecular species obtained for naturally soluble AChE in white matter and grey matter are quite similar on the basis of their sedimentation coefficient values and molecular weights.

The peaks of enzyme activity from starch block electrophoresis of sodium deoxycholate solubilized AChE and naturally soluble AChE were both resolved into a single species having a sedimentation coefficient of 11 S (M.W. of 227,000) when centrifuged on sucrose gradients. The peaks of enzyme activity (Peak I) from starch block electrophoresis of Triton solubilized AChE and naturally soluble AChE in the grey matter of porcine brain were resolved into a single species with a sedimentation coefficient of 11 - 12 S (M.W. of 240,000) (Reavill, 1976). This indicates that these molecular forms of AChE in the white matter are similar to those found in the grey matter.

The affinity chromatography purified AChE was shown to have a sedimentation coefficient of 10.6 S, both before and after starch block electrophoresis. This was the same value obtained for the crude sodium deoxycholate solubilized AChE. In the case of grey matter AChE, the affinity chromatography purified AChE was found to have a sedimentation coefficient of 11 - 12 S, before and after starch block electrophoresis. Once again, it was noted that the sedimentation coefficient values obtained for the purified AChE in both white and grey matter were fairly similar.

An interesting observation on reviewing the sedimentation coefficient values for all the different preparations was that a rather consistent sedimentation coefficient value of between 10.4 S and 11 S was obtained. This probably indicates that regardless of the method of preparation of the AChE (with the exception of using denaturing agents) the major molecular species obtained is always the same. This is supported by the results obtained from gradient gel electrophoresis (Fig. III.10) where the predominate molecular species in all the different preparations was the species with a molecular weight of 260,000 (corresponding to a 12 S species). A similar conclusion was reached by Ott et al (1975) for human erythrocyte AChE and Reavill (1976) for pig brain cortex AChE. The suggestion made by Tanford et al (1974) that membrane proteins probably retain their in vivo conformation if solubilized by mild detergents (e.g. sodium deoxycholate and Triton X-100) is probably applicable to the brain enzyme.

The results from sucrose gradient centrifugation and gradient gel electrophoresis show that in white matter AChE, a major molecular form having a sedimentation coefficient in the range 10.4 S - 12 S and a corresponding molecular weight between 209,000 and 260,000 is present. This agrees fairly well with the results obtained by Hollunger and Niklasson (1973) and Viana et al (1974) who found a species with molecular weight of 250,000 and 219,000 (for bovine brain caudate nucleus AChE), respectively.

Various brain extracts give apparent molecular weights for the predominate AChE species in the region of 80,000 - 100,000 daltons either after explicit protease treatment (Ho & Ellman, 1969) or after tissue incubation and extraction of soluble enzyme (Hollunger

& Niklasson, 1973). Other preparations of brain extracts show mainly species with apparent molecular weights of 180,000 (Jackson & Aprison, 1966a) and of 135,000 (Chan et al, 1972a). Finally, a third class of brain-extract preparations show predominantly molecular weights greater than 270,000, generally after extraction procedures which solubilize particulate acetylcholinesterase (Jackson & Aprison, 1966a; Kremzner et al, 1967; Ho & Ellman, 1969). With the addition of an apparent subunit monomer of molecular weight 85,000 - 100,000 the observations of molecular species of AChE from mammalian sources seem to be consistent with the general context defined by the enzyme in electric-organ systems. Therefore the globular subunit tetramers, dimers, and monomers appear to be present as soluble-active species and are probably generated by the action of proteases or other degradative agents on larger asymmetric particulate acetylcholinesterase.

SECTION VI

DISCUSSION

RAT MUSCLE ACETYLCHOLINESTERASE

The aim of this study was to attempt to solubilize the acetylcholinesterase in rat gastrocnemius muscle. When the enzyme was solubilized, some kinetic aspects of the AChE were studied and the various multiple molecular forms of the enzyme were examined by electrophoresis and sucrose density gradient centrifugation. A study of the inhibition of AChE by some organophosphorus compounds was also carried out.

As a result of the above investigations attempts were made to explain the relationship of the enzyme with the membrane.

1. SOLUBILIZATION

The criteria upon which the method of extraction of the AChE was adopted were as mentioned in Section V.1. As early as 1951, Ord and Thompson had used several non-ionic detergents for the solubilization of AChE. They also showed that most of the AChE from brain can be solubilized with non-ionic detergents but that ionic detergents usually yielded extracts with low or negligible activity. In addition, Jackson and Aprison (1966b) studied the effects of numerous commercial surface-active agents, including anionic, cationic and non-ionic types, on the activity of AChE in homogenates of caudate nuclei. They observed that generally, the cationic and anionic surfactants were inefficient in solubilizing AChE, but low concentrations of non-ionic surfactants (e.g. Triton X series) were effective in increasing the amount of AChE solubilized. Ho and Ellman (1969) also obtained over 85% of the AChE activity when they solubilized rat brain AChE with Triton X-100. From the above observation, it is evident that non-ionic detergents were

very efficient in the extraction of AChE and therefore in this study, Triton X-100 was used for the solubilization of rat muscle acetylcholinesterase.

A. Dilute Buffer or Aqueous Media

Extraction of rat muscle AChE with dilute buffer or aqueous solution gave between 49% and 53% of the total homogenate activity in a soluble form. (Table IV.1). When the 100,000 g. pellet from this preparation was resuspended in the buffer, homogenised and further extracted, no more enzyme was solubilized. This indicates that the naturally soluble enzyme obtained was a true soluble fraction.

B. Triton X-100

In an attempt to solubilize the particulate AChE, the non-ionic detergent, Triton X-100, was used following the method employed by Ho and Ellman (1969) with slight modifications. The results show that the detergent was very efficient in the solubilization of muscle AChE and yields between 73% and 77% of the total homogenate activity were obtained. (Table IV.2) These values are in close agreement with those of Ho and Ellman (1969) who obtained over 85% of the total activity when they solubilized rat brain AChE with 0.5% (w/v) Triton X-100. The relatively high yield of enzyme obtained suggests that the muscle AChE is not strongly associated with the membrane and hence the use of Triton X-100 is successful for solubilizing the enzyme.

Increasing the Triton X-100 concentration in the extraction medium caused an increase in the amount of AChE solubilized, with a maximal effect at 2% (w/v) Triton X-100 (Fig. IV.1). Similar results were obtained by McIntosh (1973) for porcine brain AChE.

When the AChE activity in the naturally soluble fraction was summated with the activity in the Triton-solubilized fraction, a value corresponding to 126% of the total homogenate activity was obtained. Therefore the results suggest that some activation of the AChE had occurred. This was demonstrated when a 15% - 25% activation of AChE activity was observed when the enzyme activity of the uncentrifuged homogenate was assayed in the presence of 1% (w/v) Triton X-100.

Several workers have also found that Triton X-100 activated the AChE activity. Jackson and Aprison (1966b) found that Triton increased the AChE activity apart from solubilizing it, because activation was found at a low concentration of Triton which did not solubilize the enzyme. This effect of Triton on AChE could not be repeated by Crone (1971) but he reported that activation was always associated with solubilization of AChE and that it depended on the Triton to protein ratio rather than the Triton concentration per se. However, Crone stated that this was only critical at the lower range of Triton concentrations used when solubilization of the enzyme was incomplete and activation was constant over a wide range of the Triton to protein ratio (2 - 100 mg. Triton/mg. protein) at 'complete' solubilization of AChE. Srinivasan et al (1972) also reported about the activating effect of Triton on brain AChE. They measured the specific AChE activity in the supernatant and precipitate after ultracentrifugation (100,000 g. for 1 h.) of a homogenate treated with Triton and they found that different concentrations of Triton (0.2 - 5%) resulted in an increase in the supernatant AChE activity while the activity of the precipitate remained virtually constant. Therefore they concluded that Triton not only solubilized

the enzyme but also directly activated its catalytic activity. However, uncertainty in their experimental method made their conclusion unfavourable. They expressed AChE per mg. protein in the fraction; AChE and total protein could well be solubilized differentially, which might explain the findings. Moreover, as can be calculated from their data, the total protein of the homogenate increased as a function of increasing Triton concentrations; perhaps no correction was made for the interference of Triton with the Lowry protein assay. Raaijmakers (1978) carried out some experiments on the effect of Triton on AChE and he showed that (i) freezing of tissue before homogenization led to an increase in AChE activity in the homogenate and this he said was the major cause of the activation of AChE, (ii) Triton did not alter the K_m but only the V_{max} of AChE in the homogenate or in any particulate fraction, indicating that no conformational change of the active centre of AChE had occurred but that as more enzyme is solubilized, more of it is exposed to the substrate, (iii) age differences affect activation which cannot be explained by freezing or storage of samples and maximal activation is found in brains of rats of 3 - 4 weeks old. The results obtained in this study show that Triton causes activation of the AChE activity in the homogenate which is in agreement with the findings of Skangiel-Kramska and Niemierko (1975) and Raaijmakers (1978) who both found that the activating effect of Triton depended on the particle-bound state of the AChE, while working on AChE in peripheral nerves of vertebrates and rat brain AChE, respectively.

In spite of the activation effect of Triton on AChE which has been observed in this study, the advantages of the use of the detergent exceed the activation effect. The advantages are that Triton

does not induce conformational changes in proteins which may lead to a loss in their biological activity (Meunier et al, 1972; Rubin & Tzagoloff, 1973) and Triton is inefficient in disrupting protein-protein interactions (Helenius & Simons, 1975).

2. KINETIC PROPERTIES

The kinetic parameters investigated in this study include the pH dependence, the substrate dependence, the specificity, Michaelis constants and Arrhenius plots of the muscle preparations.

The pH-activity profiles and the substrate-velocity curves of the solubilized AChE (Figs. II.4 and II.5) were similar to results obtained for AChE from other sources, having a pH optimum in the region pH 7.6 - 8.3 and a substrate optimum in the range 1 - 2 mmol/l. For AChE, a pH optimum value of 8.25 (8.0 - 8.5) is usually reported (Cohen & Oosterbaan, 1963; Silver 1974) although sometimes lower values are found, even if the same substrate is used (Varela, 1973).

The specificity of the enzyme under normal assay conditions is that of a specific acetylcholinesterase since maximum activity was obtained with acetylcholine and acetylthiocholine as substrates (Table IV.3). In addition, the hydrolysis of acetyl-β-methylcholine and the phenomenon of enzyme inhibition at high substrate concentration both corroborate this conclusion. The low hydrolysis rate for butyrylcholine indicates the presence of a cholinesterase which has also been reported in other species.

The Michaelis constant for the AChE in the crude homogenate was found to be 84 µmol/l and that of the naturally soluble AChE was 110 µmol/l (Table IV.4). These values are higher than those

obtained for rat brain AChE which were 71 μmol/l and 67 μmol/l, respectively (Raaijmakers, 1978). The explanation for the difference may be that the AChE is from different sources of tissue and generally, brain AChE has been observed to have a lower K_m value, indicating a higher affinity of the enzyme for its substrate, than the muscle AChE. The K_m value for the Triton solubilized enzyme was found to be 72 μmol/l, which was the lowest value obtained for the preparations examined. This suggests that solubilization of the AChE may have increased the affinity of the solubilized enzyme for its substrate. A similar result was observed with the porcine brain AChE.

The Arrhenius plots of AChE in the membrane preparation showed a definite break at 26°C (the transition temperature). The activation energies for the enzyme on either side of this temperature were different, being 21 $kJmol^{-1}$ from 25°C - 40°C and 35 $kJmol^{-1}$ from 10°C - 25°C. Several reasons may be given for the existence of such phase changes at specific temperatures for enzymes and Dixon & Webb (1964) have cited several explanations for such phase changes. Phase changes have been observed with lipase, and with sucrase which exists in two forms of differing activities and the enzyme changes from one form to another at 22°C. The results in this study show that the Triton solubilized AChE and the naturally soluble AChE did not show any phase changes and the estimated activation energies are 24 $kJmol^{-1}$ and 9.6 $kJmol^{-1}$, respectively, over the temperature range 10°C - 40°C.

It is very likely that the phase change observed with the membrane preparation AChE depended upon the particulate nature of the enzyme. However, this conclusion is not unequivocal.

Taylor (in Plummer et al, 1975) suggested that the discontinuity in the Arrhenius plots represented a local structural change in the membrane rather than a true phase transition of the integral membrane. He said that transitions occurring over a wide temperature range would be the likely situation for membranes of heterogeneous composition or those containing associated proteins. This explanation seems to apply to the AChE in rat muscle.

Phase changes for soluble AChE have been reported by Ciliv and Özand (1972) who studied the Arrhenius plot of erythrocyte AChE and found a phase transition at 32°C. They suggested that the break in the Arrhenius plot was probably due to a change in the aggregation state of the enzyme or by a reversible change of enzyme protein between two states, indicating that one of the states was more active at temperatures above 32°C. Such phase changes were not observed for the soluble AChE of rat muscle (Figs. IV.3 and IV.4). The difference in results may be attributed to the difference in the source of the enzyme.

3. MULTIPLE MOLECULAR FORMS OF MUSCLE ACETYLCHOLINESTERASE

Acetylcholinesterase is found in multiple forms in tissues other than nerve and brain, and of these the highest specific activities are found in muscle and erythrocyte membranes. Maynard (1966) found three electrophoretic forms of AChE in chick muscle and Wilson et al (1970) found embryonic forms which are retained only in the muscular dystrophic chick and not in the normal adult muscle. As mentioned earlier for the porcine brain AChE, it is likely that the method of preparation of the enzyme from a membrane-bound location is an important factor in determining the different

molecular forms obtained.

The methods used for the separation of the molecular forms of AChE in this study are starch block electrophoresis, gradient polyacrylamide gel electrophoresis and sucrose density gradient centrifugation.

A. Starch Block Electrophoresis

When the Triton solubilized AChE was electrophoresed on a starch block with detergent excluded from the block, it was resolved into one peak of enzyme activity with a very low negative mobility and was located near the origin. However, when Triton X-100 was present in the starch block, the Triton solubilized AChE was resolved into two peaks of enzyme activity (Fig. IV.5). Since the exclusion of Triton X-100 in the starch block caused the enzyme to resolve into a single peak of very low negative mobility, this suggests that the lack of detergent led to aggregation of AChE. This conclusion was supported by the observation that when Triton X-100 was included in the starch block, the enzyme was resolved into two peaks. The slow moving peak (Peak II) was found to be an aggregated form of the AChE while Peak I was resolved into several molecular forms when they were electrophoresed on polyacrylamide gel.

The naturally soluble AChE was resolved into two distinct peaks of enzyme activity, even in the absence of Triton X-100 (Fig. IV.6). Similarly as for the Triton solubilized enzyme, the slow moving peak (Peak II) was shown to be in an aggregated state while the faster moving peak (Peak I) was found to separate into multiple molecular forms when electrophoresed on gradient polyacrylamide gel.

In these studies the Triton solubilized enzyme seemed to have a slightly greater negative mobility than the naturally soluble AChE. This may be due to the different methods of extraction of the AChE or alternatively, the Triton X-100 may have affected the mobility of the enzyme, but these are merely tentative speculations.

Generally, the results obtained by this separation technique indicated that the molecular forms of AChE did not arise from differences in their charges but from differences in molecular weights as demonstrated by polyacrylamide gel electrophoresis.

B. Gradient Polyacrylamide Gel Electrophoresis

Multiple bands of AChE activity (9 - 11 bands) were obtained for the different enzyme preparations using this method. The AChE staining pattern for the molecular forms are shown in Fig. IV.7. The electrophoretograms show that the major species found in the enzyme preparations are species having molecular weights of approximately 100,000, 355,000 — 394,000, 521,000 and 733,000 daltons. Similar molecular weight species have been obtained by sucrose density gradient centrifugation corresponding to molecular weights of 92,000, 399,000 and 558,000 daltons. In addition to these species, the Triton solubilized AChE had another predominate molecular form with a molecular weight of 225,000 daltons.

Some of the molecular species obtained are similar to the aggregated forms of AChE in mouse brain obtained by Adamson et al (1975), which had molecular weights of 360,000 and 720,000 daltons. Adamson et al (1975) found that the lowest molecular weight form (monomer) of the mouse brain enzyme was a species of 74,000 daltons. However, in these studies, the lowest molecular weight form obtained for the different enzyme preparations was a 100,000 daltons

species. Several reports on AChE species have stated that dimers were found with a range of molecular weights from 102,000 —134,000 daltons (Table I.2). It is therefore tempting to speculate that the 100,000 dalton species is a dimer and the other predominate species with molecular weights of 355,000 —394,000, 521,000 and 733,000 daltons are different multiples of the subunit monomer which may have a molecular weight of 50,000 daltons. Levinson and Ellory (1974) found that AChE from electric eel and erythrocytes behaved predominantly as a dimer when solubilized by Triton X-100.

From the results obtained from the electrophoretograms, it is interesting to note that the range of molecular weights for both the naturally soluble and Triton solubilized AChE are very similar, ranging from 100,000 —733,000 daltons. In general, it may be concluded that the different methods of extraction of AChE have yielded molecular forms which are very similar and this indicates that they are true molecular species and not artefacts of preparation.

Gradient electrophoresis of the enzyme peaks eluted from starch block electrophoresis of both enzyme preparations showed that Peak II (the slow moving peak) just entered the polyacrylamide gel while Peak I (the fast moving peak) showed a similar AChE staining pattern as the crude enzyme. This result suggests the presence of both an aggregated and non-aggregated population of multiple molecular forms. In addition, since the non-aggregated Peak I AChE separated into various molecular forms after electrophoresis on a polyacrylamide gradient, the differences in molecular forms could be attributed to differences in molecular weight rather than charge.

C. Sucrose Density Gradient Centrifugation

The Triton solubilized AChE when centrifuged on sucrose gradients incorporated with 1% (w/v) Triton X-100 was resolved into three peaks of enzyme activity, corresponding to species having sedimentation coefficients of 8.5 S (M.W. 155,000), 16 S (M.W. 399,000) and 20 S (M.W. 558,000) (Fig. IV.8). The 8.5 S species may be similar to the dimeric globular form designated as form G'_s, obtained as a result of sonication and/or solubilization with Triton X-100 by Massoulié et al (1971) and Millar et al (1973) (Table I.3). The 16 S and 20 S species may represent assemblies of subunits forming either globular or elongated forms of AChE. Grafius and Millar (1965, 1967) showed that even after prolonged toluene treatment, molecular forms of AChE having sedimentation coefficients of approximately 14 S and 18 S and which aggregated to 65 S particles when dialyzed against buffers of ionic strength below 0.1, were obtained. Massoulié extended this work and he showed that membrane-bound AChE, in the absence of proteolytic solubilization procedures, can be obtained in highly asymmetric forms termed A, C and D. In electron microscopy these forms appeared as grape-like structures, being composed of a tail with clusters of AChE subunits attached to it (Rieger et al, 1973a).

The naturally soluble AChE was resolved into two peaks of enzyme activity when centrifuged on a sucrose gradient (Fig. IV.10). The corresponding species obtained were found to have sedimentation coefficient values of 6 S (M.W. 92,000) and 16 S (M.W. 399,000). The molecular species obtained for both naturally soluble AChE and Triton solubilized AChE by sucrose gradient centrifugation correlate well with the results obtained by gradient gel electrophoresis.

Molecular forms having molecular weights of approximately 100,000 daltons, 394,000 daltons and 521,000 daltons were found when either separation technique was used.

Peak II (the slow moving peak) from starch block electrophoresis of naturally soluble AChE and Triton solubilized AChE migrated to the bottom of the tube when centrifuged on a sucrose gradient. This indicates that a highly aggregated form of AChE is present in Peak II. However, Peak I from starch block electrophoresis of both AChE preparations was resolved into a single peak of enzyme activity, corresponding to a 11 S species (M.W.227,000) (Figs. IV. 9 and IV.11). This molecular form was also obtained by gradient gel electrophoresis of naturally soluble AChE and Triton solubilized AChE where a species having a M.W. in the range 225,000 - 282,000 was found.

Ott et al (1975) obtained a rather anomalous result where the Triton solubilized enzyme 'floated' in the sucrose gradient and hence giving an apparently low sedimentation coefficient value. They explained their results by suggesting that the protein molecule probably combined with detergent molecules and caused the enzyme to float in the sucrose. In addition, Tanford et al (1974) suggested that Triton X-100 is probably bound to lipophilic regions on the enzyme and therefore increases the partial specific volume. This floating phenomenon was not observed in these studies and a probable explanation is that the muscle enzyme did not bind to many detergent molecules.

Hall (1973) has carried out experiments to investigate the molecular species of AChE in rat diaphragm and observed three principal forms, the 4 S, 10 S and 16 S species. Hall stated that

the 4 S species appeared to be a soluble in vivo species, while the 10 S and 16 S species were probably membrane-bound. The 16 S AChE species was found to be specifically associated with the endplate regions of muscle and was greatly reduced in amount upon denervation. Vigny et al (1976a,b) provided additional evidence suggesting that the heaviest form (H) was endplate-specific in various muscles of the rat and chicken. In the studies carried out by Hall and Vigny et al, the H form accounted for only a minor part of the total AChE activity, the greater part of the enzyme occurring in peaks of medium (M) or low (L) sedimentation coefficient. Similar results were obtained in this study. In the Triton solubilized AChE, the 16 S and 20 S species (H forms) were found to account for a small part of the total AChE activity while the 8.5 S species (M form) formed the major part of the total activity; and in the naturally soluble AChE, the 16 S species (H form) was also found to contribute a small part to the total AChE activity while the 6 S species (M form) accounted for the greater part of the total activity.

The 16 S AChE species found in skeletal muscle segments containing neuromuscular junctions may be referred to as an 'endplate specific' form, but it clearly does not represent all activity at the endplate. This species disappeared after denervation (Hall, 1973; Vigny et al, 1976b) and reappeared following reinnervation either at the original endplate or at ectopic sites. Vigny et al (1976b) demonstrated that it took several days for the disappearance of the 16 S form after denervation,therefore suggesting a post-synaptic localisation. Koenig and Vigny (1978) showed that in posterior leg muscles from rat embryos, the 16 S form was absent before the 14th day of gestation and it appeared at day 15 and was

relatively more abundant on day 16 (25% of the total AChE activity) than in the adult (5%). Vigny et al (1976b) also found that the total AChE activity increased by a factor of 10^3 from day 16 of gestation to the adult muscle. Koenig and Vigny (1978) demonstrated that the 16 S form may be produced by muscle cell cultures on induction by neuronal elements. In addition, studies of neuroblastoma cell cultures have shown that the 4 S form is a precursor of the 10 S form. The results obtained by Koenig and Vigny (1978) also suggest that these forms might in turn be precursors of the 16 S molecule.

Various skeletal muscles in chicken were found to contain a molecular form with a sedimentation coefficient of 19.5 S (Vigny et al, 1976a). The 20 S AChE species found in the Triton solubilized enzyme in this study may be a very similar molecular species to this 19.5 S species. Vigny et al (1976a) demonstrated that after denervation, the high molecular weight 19.5 S species (H form) disappeared from crude extracts of muscle. As the H form was only found in tissues innervated by cholinergic nerve endings and disappeared or decreased drastically from denervated skeletal muscles, it has been suggested that this form of AChE is exclusively myogenic (Hall, 1973; Rieger & Vigny, 1976) and may constitute the specific endplate enzyme (Hall, 1973; McLaughlin & Bosmann, 1976; Vigny et al, 1976a,b).

Several workers have demonstrated that treatment with collagenase or proteases can modify the molecular forms of AChE in skeletal muscle (Hall & Kelly, 1971; Silman et al, 1978; Bon et al, 1978). Silman et al (1978a) and McLaughlin & Bosmann (1976) suggested that endogenous proteases may affect the distribution of

AChE species in skeletal extracts. Silman et al (1978b) examined the effect of certain protease inhibitors (EGTA, benzamidine hydrochloride, N-ethylmaleimide, leupeptin, pepstatin and bacitracin) on the sedimentation profile of AChE activity in extracts of various chicken muscles and of rat diaphragm. They showed that when sucrose gradient centrifugation was performed on extracts of the endplate regions of rat diaphragm, in the presence and absence of protease inhibitors, two very different sedimentation profiles were obtained. In the absence of the inhibitors the H form (16 S) was a minor component compared to the L form (3.4 S) and M form (9.7 S), whereas in the presence of protease inhibitors it was the major form obtained. In addition, in the presence of protease inhibitors, heavier components in both the M and L zones of the gradient predominate, whereas lighter forms were more predominant in both these zones in the absence of protease inhibitors. These results demonstrate that the use of suitable protease inhibitors in the preparation of muscle extracts markedly retards the modification of intrinsic forms of AChE present in the tissue. They also show that when a given muscle type from an adult is examined under conditions where degradation is largely prevented, the distribution of molecular forms is, in fact, much simpler than was thought previously, and a single molecular form usually predominates. From these observations, it is essential that considerable care must be taken in analysing the distribution of AChE forms in any tissue and in assigning functional significance to all of the species obtained.

4. INHIBITION OF ACETYLCHOLINESTERASE BY ORGANOPHOSPHORUS COMPOUNDS

The results obtained for the naturally soluble AChE and Triton solubilized AChE showed that the bimolecular rate constants (k_i)

were the same whether or not substrate was present during the inhibition of AChE (Tables IV.6 and IV.7). This indicated that organophosphate inhibition of acetylcholinesterase was non-competitive with regard to the substrate. However, the assumption of purely competitive kinetics is inherent in the arguments of Main and Dauterman (1963) concerning the determination of bimolecular rate constant, k_i, for the inhibition of AChE by an organophosphate in the presence of substrate. They demonstrated that the k_i values were in excellent agreement with those determined in the absence of substrate. Therefore, they suggested that organophosphates generally react only with the free active centre of the enzyme and this conclusion is supported by Dixon and Webb (1964). The results obtained in these studies seem to agree favourably with this conclusion.

The bimolecular rate constants obtained for the naturally soluble AChE were found to be lower than those obtained for the Triton solubilized AChE. This suggested that the naturally soluble AChE was less susceptible to inhibition by organophosphates than the Triton solubilized AChE. This may be due to the removal of some factor during solubilization of the membrane-bound AChE which made the detergent solubilized enzyme more prone to inhibition in the presence of organophosphates.

The results, however, do not show a similar pattern for the inhibitory effect of the organophosphorus compounds on the naturally soluble AChE and the Triton solubilized AChE. Although the results demonstrated that ethyl malaoxon was the best inhibitor for the naturally soluble enzyme, that methyl paraoxon was the best inhibitor for the Triton solubilized AChE and that isopropyl paraoxon was

the least effective inhibitor for both enzymes, no other conclusions can be made concerning the behaviour of the enzyme preparations in the presence of these inhibitors.

Devonshire (1975) studied the inhibition of AChE from heads of insecticide-resistant and -susceptible houseflies (Musca domestica L.) by organophosphorus insecticides. He found that the bimolecular rate constants for inhibition (k_i) were the same whether or not substrate was present during inhibition, for both strains of houseflies, which was in agreement with the results obtained in this study. He also observed that the rate of inhibition by malaoxon was lower in the presence of Triton X-100, and this effect was greater on the susceptible enzymes. This suggests that the Triton solubilized AChE in houseflies is less susceptible to inhibition than the enzyme in the absence of Triton X-100. From these results it can be seen that the Triton solubilized AChE in houseflies and in rat muscle behave differently in the presence of organophosphates and this may be explained by the difference in the source of the enzyme.

The membrane protein classification developed by Singer (1974) is operational and is based on the way these proteins can be detached from the membrane. The peripheral proteins are relatively readily dissociated from the membrane (using chelating agents; by lowering or increasing the ionic strength or the pH) without disrupting the lipid matrix of the membrane, whereas the integral proteins are tightly bound to the membrane and can only be solubilized by disrupting the membrane with more drastic treatment (use of organic solvents or detergents). Another characteristic of integral

proteins is that they usually aggregate or are not soluble in neutral aqueous buffer. The results obtained in this study showed that sodium deoxycholate and Triton X-100 were the most efficient solubilizing agents for AChE in the white matter of porcine brain and AChE in rat gastrocnemius muscle, respectively. In addition, the phenomenon of aggregation was observed in the enzyme of both tissues. These findings, therefore, led to the conclusion that AChE in both white matter of porcine brain and rat gastrocnemius muscle was an integral protein.

The presence of acetylcholinesterase in axons has posed a problem in terms of the classical neurohumoral transmitter theory and Nachmansohn has proposed that AChE is actively involved in the propagation of the nerve impulse along the axon as well as at the synapse (Nachmansohn & Neumann, 1975). Another possibility is that the AChE found in the axon is transitory in nature and it may eventually be incorporated in the membrane at the synapse. However, the apparent tight binding of the AChE in the white matter would suggest that this is not possible and more extensive study will be required to determine the precise role of axonal AChE with regard to nerve impulse transmission. An interesting study would be to reconstitute axonal AChE into model membranes and observe its behaviour under those conditions. This may help to clarify its role in the axon and either prove or disprove Nachmansohn's theory that chemical processes are involved in the conduction of nerve impulses along the axon.

SECTION VII

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Electrophoretic techniques for separating the multiple molecular forms of acetylcholinesterase from porcine brain

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Previous work in our laboratory on the enzyme acetylcholinesterase (acetylcholine hydrolase, E.C. 3.1.1.7) has shown that about 15% of the enzyme from human erythrocytes and pig brain cortex can be extracted with simple aqueous media and this enzyme is referred to as the 'naturally soluble' form. This present study compares the soluble and membrane forms of the enzyme present in the grey and white matter of brain. This 'naturally soluble' form was obtained by extracting the tissue with water and the membrane bound form was brought into solution with detergents. Triton X-100 was found to be the best detergent for grey matter and at a concentration of 1% (w/v) about 56% of the total activity was brought into solution. Deoxycholate on the other hand was by far the best solubilizing agent for the acetylcholinesterase present in the white matter when about 69% was solubilized at a concentration of 0·2% (w/v) of the detergent.

We compared and demonstrated the differences in the multiple molecular forms of acetylcholinesterase present in the soluble and membrane fractions of the grey and white matter of pig brain. The main techniques demonstrated were electrophoresis on a starch block (Plummer, Elliott, Cooke & Wilkinson, 1963; Leathwood & Plummer, 1970) and electrophoresis on a gradient of polyacrylamide (McIntosh & Plummer, 1973). In the former case, enzyme molecules are separated on the basis of differences in their charge and in the latter case separation occurs on the basis of differences in molecular weight or size.

In the application of these techniques, starch-block electrophoresis of the 'naturally soluble' form of the enzyme from the cortex gave two peaks of activity. The slower migrating peak was shown to be a high molecular weight aggregated form of the enzyme, by electrophoresis on a gradient of polyacrylamide and centrifugation on a sucrose density gradient (Martin & Ames, 1961). The faster migrating peak on the other hand consisted of unaggregated enzyme with a range of molecular weights. Identical results were obtained with the membrane form of acetylcholinesterase solubilized with 1% (w/v) Triton X-100 provided that detergent was included in the block and tank buffer solution. If the Triton X-100 was omitted from the electrophoresis medium then only the aggregated form was obtained.

[P.T.O.

In contrast to the above findings, the acetylcholinesterase from white matter gave only one peak of unaggregated enzyme for the 'naturally soluble' and deoxycholate solubilised enzyme with detergent in the starch block.

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